IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	SCHNEIDER, Michel et al.	Group Art No. 1619
Reissue of Patent No.:	5,413,774	
Issued:	May 9, 1995	
Appl. No.:	09/115,963	
Filed:	July 15, 1998	
FOR:	LONG LASTING AQUEOUS DISPERSIONS OR SUSPENSIONS OF PRESSURE-RESISTANT GAS-FILLED MICROVESICLES AND METHODS FOR THE PREPARATION THEREOF	
Examiner:	M. Hartley	
Attorney Docket No.:	DUP-0478	

#21
AKO
6-26-01

TRANSMITTAL OF PROTEST UNDER 37 CFR §1.291(a)

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Enclosed for filing in the above-identified Patent Application are the following documents:

(1) Protest Under 37 CFR 1.291(a);

(2) Exhibit 1: U.S. Patent No. 5,393,524 to Quay, filed June 4, 1993, and granted February 28, 1995;

(3) Exhibit 2: WO 91/15244, filed April 2, 1991;

(4) Exhibit 3: EP 0458745A1, filed May 14, 1991; and

(5) a return pre-paid postcard.

Protester authorizes the Commissioner to charge any additional fees which may be required to Account No. 23-3050.

Respectfully submitted,

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re **REISSUE APPLICATION** of:

Michel Schneider, et al.

Serial No.: **09/115,963** Group Art Unit: **1619**

Filed: **July 15, 1998** Examiner: **M. Hartley**

(Reissue Application of U.S. Patent No. 5,413,774, Granted May 9, 1995)

For: **LONG LASTING AQUEOUS DISPERSIONS OR SUSPENSIONS OF PRESSURE-RESISTANT GAS-FILLED MICROVESICLES AND METHODS FOR THE PREPARATION THEREOF**

Assistant Commissioner for Patents
Washington, D.C. 20231

PROTEST UNDER 37 CFR §1.291(a)

This is a protest, on behalf of the DuPont Company ("Protestor"), pursuant to 37 C.F.R. §1.291(a), against the patentability of all claims of the above-identified application. The above-identified application is a reissue application ("Reissue Application") of United States Patent No. 5,413,774 granted on May 9, 1995 ("the '774 patent"). As this protest is being filed prior to the mailing of a notice of allowance, Protestor respectfully requests that this protest be entered in the above- identified application in accordance with 37 C.F.R. §1.291(a)(1). This protest, including all accompanying exhibits, has been served on Applicants' attorney in accordance with 37 C.F.R. §1.291(a)(2).

This is the second protest submitted by Protestor against the above-identified application. Protestor is cognizant of the admonition in 37 C.F.R. § 1.291(c) that no further submission on behalf of a protestor will be considered except for additional prior art, or unless such submission raises new issues which could not have been earlier presented. Protestor submits, however, that this provision does not preclude the filing of the instant Protest in view of the filing by Applicants of a Request for Continued Prosecution Application (CPA), on

December 7, 2000. As set forth in 37 C.F.R. § 1.53(d)(2)(v), the filing of a CPA constitutes an express abandonment of the previous application. Protestor submits, therefore, that since Applicants have expressly abandoned the previous application, the instant Protest should be treated as a "new" protest in a "new" application. Attention is directed in this regard to MPEP § 1901, where it is stated that "[a]ny protest filed in a continuing application is treated as a new protest."

I. GROUNDS FOR PROTEST

Protestor respectfully submits that currently pending Claims 1 to 3, 7, 13 to 22, 26 and 30 to 48 of the above identified application are unpatentable under 35 U.S.C. § 103 over Quay, U.S. Patent No. 5,393,524 ("Quay"), in view of Schneider et al., WO 91/15244 ("Schneider"), and/or Bichon, et al., EP 0 458 745 A1 ("Bichon").

These grounds for protest are discussed in greater detail hereinafter. Protester requests that the Examiner consider these grounds and find that the claims of the above identified application are unpatentable.

II. INFORMATION ON WHICH THE PROTEST IS BASED

Protestor hereby submits the following Exhibits upon which this protest is based. Included are prior art documents that render Applicants' reissue claims in the above identified application unpatentable.

A. Prior Art Documents

Exhibit 1 U.S. Patent No. 5,393,524 to Quay, issued February 28, 1995, based on U.S. Application Serial No. 71,377, filed June 4, 1993, continuation of Serial No. 761,311, filed September 17, 1991, abandoned.

Exhibit 2 WO 91/15244 to Schneider et al., filed April 2, 1991, published October 17, 1991

Exhibit 3 EP 0 458 745 A1 to Bichon et al., filed May 14, 1991, published November 27, 1991.

III. PROCEDURAL HISTORY

Applicants Schneider et al., seek reissue of United States Patent No. 5,413,774 ("the '774 patent") granted on May 9, 1995. The '774 patent issued from United States Patent Application Serial No. 07/991,237 ("the '237 application"), filed December 16, 1992. The '237 application, when originally filed, claimed priority under 35 U.S.C. §119 to European Patent Application EP 92810046.0 ("EP '046"), filed January 24, 1992[1].

As discussed in depth in the Protest dated April 13, 2000, at the time of filing the reissue application, Applicants added new claims of priority to two previously unrelated lines of cases, identified in that Protest as the Microbubble and Microballoon Application Families, in an attempt to improperly gain benefit of the filing dates of the earliest of those applications. This was no doubt due, at least in part, to the fact that absent the benefit of those earliest filing dates, publications of applications in those families, WO 91/15244 ("Schneider") and EP 0 458 745 A1 ("Bichon"), respectively, are prior art to the '774 patent and the instant application.

In Office Actions dated September 29, 1999 and August 29, 2000, and reiterated again in the latest Office Action dated March 28, 2001, the Patent Office correctly denied this claim to priority, *inter alia*, because the earlier applications do not provide written description support for the subject matter claimed, in the manner required by 35 U.S.C. §§ 119 and 120. Indeed, missing from the disclosure of these prior applications is an identification of the particular gases presently being claimed by Applicants. Accordingly, the effective filing date of

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[1] Protestor notes that the filing date of EP '046 was incorrectly noted on the '774 patent as being January 23, 1992.

the instant application can be no earlier than January 24, 1992, and as such, the Schneider and Bichon publications remain available as prior art to this application.

IV. CLAIM ANALYSIS

Claims 1 to 3, 7, 13 to 22, 26, and 30 to 48 are currently pending in this application. Claims 1, 2, 13, 15 to 20 and 32 to 34 are independent claims. In general, the claims are directed to methods for making contrast agents for ultrasonic echography. The contrast agents consist of gas-filled microvesicles that are either *gas-filled microbubbles* or *gas-filled microballoons*. The term "microbubbles" is defined as hollow spheres or globules, filled with air or a gas in suspension in a liquid, having only "an immaterial or evanescent envelope." See the '774 patent, column 1, lines 27 to 33 and column 2, lines 20 to 22. The term "microballoons" is defined as air or gas-filled bodies with a material boundary or envelope. *See* '774 patent, column 1, lines 33 to 36. The currently pending claims further define these entities, and distinguish them from one another, by specifying that the claimed microbubbles are "bounded by a stabilizing layer of one or more film forming phospholipids in lamellar or laminar form at the gas/liquid interface" (*see* Claims 1 to 3, 7, 13 to 15, 18, 21, 26, 32, 35, and 37 to 42), while the microballoons have an organic polymer envelope "formed from one or more polymers selected from the group consisting of polylactic or polyglycolic acid and their copolymers, denatured albumin, reticulated hemoglobin, and esters of polyglutamic and polyaspartic acids" (*see* Claims 16, 17, 19, 20, 22, 30, 33, 34, 36, and 43 to 48).

The microvesicles are defined in *all claims* as containing a "physiologically acceptable gas selected from the group consisting of SF_6, CF_4, $CBrF_3$, C_4F_8, $CC1F_3$, C_2F_6, C_2C1F_5, $CBrC1F_2$, $C_2C1_2F_4$ and C_4F_{10}". Claims 15, 17, 18, 20, 21, 22, 26, 30 to 32, 34 to 36, and 43 further define that the aforementioned physiologically acceptable gas is present as part of a *gas mixture*. The claimed methods entail either forming the microvesicles under an atmosphere containing one of the aforementioned gases or gas mixtures (Claims 1, 7, 13, 14, 15, 16, 17, 26 and 30 to 48), or preforming the microvesicles or precursors thereof under an atmosphere of a first gas and substantially substituting at least a fraction of the first gas with one of the physiologically acceptable second gases (Claims 2 and 18 to 22).

All claims also require that the microvesicles (i.e., either the microbubbles or microballoons) be suspended in an aqueous liquid carrier phase and be *resistant against collapse from pressure increases* that are effective when a suspension of the gas-filled microvesicles is injected into the bloodstream of a patient. Applicants teach that the resistance against collapse is achieved through the selection of the gas (*see, e.g.,* abstract and column 3, line 65 to column 4, line 4). Therefore, the resistance against collapse is an inherent property of the particular gases selected by Applicants. Similarly, in certain of the claims it is stated that the physiologically acceptable gas is such that, under standard conditions, the pressure difference between pressures at which the bubble counts are about 75% and 25% of the original bubble count is at least 25 Torr (independent Claims 13, 32, 33, and 34). Once again, this property is conferred by the selected gas itself, and is thus an inherent property of the gas selected.

The dependent claims further define such aspects as methods for forming the microballoons (Claims 30 and 31); compositions for the aqueous liquid carrier containing microbubbles (Claims 7 and 26); preferred gases used to form the microbubbles and microballoons (Claims 37 to 48); and preferred properties of the gases used to form the microbubbles and microballoons (Claims 3, 14, 21, 22).

VII. APPLICANTS' CLAIMS ARE UNPATENTABLE

Pending Claims 1 to 3, 7, 13 to 22, 26 and 30 to 48 of the reissue application are unpatentable under 35 U.S.C. § 103 over Quay, in view of Schneider and/or Bichon.

A. Summary of the Law

A patent claim is invalid pursuant to 35 U.S.C. § 103 if the subject matter that it defines would have been obvious, to one of ordinary skill in the art, at the time of filing of the application.[2] In determining whether a claim is invalid under Section 103, the claimed invention

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[2] This section of Title 35 U.S.C. provides, in pertinent part:

must be considered as a whole. *Kimberly-Clark Corp. v. Johnson & Johnson*, 745 F.2d 1437, 1448, 223 USPQ 603, 609 (Fed. Cir. 1984). In analyzing prior art references as a whole, the issue to be resolved is whether they "would have suggested [the] invention to one of ordinary skill [in the art] at the time the invention was made." *In re Merck & Co., Inc.*, 800 F.2d 1091, 1097, 231 USPQ 375, 379 (Fed. Cir. 1986).

When references are combined to support an obviousness argument, there must be a suggestion or teaching to make the combination. However, the "suggestion to modify the art . . . need not be expressly stated in one or all of the references used to show obviousness." *Cable Elec. Prods., Inc. v. Genmark, Inc.*, 770 F.2d 1015, 1025, 226 USPQ 881, 886 (Fed. Cir. 1985). "Rather, the test is what the combined teachings of the references would have suggested to those of ordinary skill in the art." *Id.* (citing *In re Keller,* 642 F.2d 413, 425, 208 USPQ 871, 881 (C.C.P.A. 1981)). It is proper, therefore, to rely on "knowledge clearly present in the prior art" to support the obviousness argument with the suggestion to make the combination inherent from this common knowledge. *See id.*; *In re Sernaker,* 702 F.2d 989, 995, 217 USPQ 1, 6 (Fed. Cir. 1983). Generally, when the elements appear in the prior art in the same configurations serving the same functions to achieve the same results as suggested by the prior art, the claims will be held to be obvious. *In re Gorman,* 933 F.2d 982, 987, 18 USPQ 2d 1885, 1888 (Fed. Cir. 1991); *Ryco, Inc. v. Ag-Bag Corp.*, 857 F.2d 1418, 1425, 8 USPQ 2d 1323, 328-9 (Fed. Cir. 1988). Moreover, where the only difference between the claimed invention and the prior art is some range or other variable within the claims, "the applicant must show that the particular range is *critical*, generally by showing that the claimed range achieves unexpected results relative to the prior art range." Otherwise, the claims will be deemed obvious. *In re Woodruff,* 919 F.2d 1575, 1578, 16 USPQ 2d 1934, 1936 (Fed. Cir. 1990).

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§ 103 Conditions for patentability; non-obvious subject matter

A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which the subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

B. Summary of Quay

Quay relates to agents that are disclosed as enhancing the contrast in an ultrasound image. *See* Quay, col. 1, lines 9 to 10. More specifically, Quay describes methods of selecting gases that are disclosed as being suitable for use in preparation of microbubble contrast enhancing media. *See* Quay, col. 10, lines 10 to 21. This method identifies gases that are particularly long-lived in solution. *See* Quay, col. 12, line 67 to col. 13, line 1. Quay indicates that microbubbles of the gases selected therein will survive long enough to permit imaging of the left ventricle, myocardial perfusion, and dynamic organ imaging. *See* Quay, col. 15, lines 1 to 18.

Quay states that bubble stability, and hence life-span, is enhanced by selecting a gas having high density, low solubility, and a low coefficient of diffusivity in the liquid phase. *See* Quay, col. 11, lines 17 to 22. Quay goes on to explain that diffusivity is dependent upon the molar volume of the gas, which is in turn correlated with the molecular weight of the gas. *See* Quay, col. 11, lines 23 to 30. Thus, Quay teaches that gases having high density, high molecular weight, and low solubility, would be potentially useful for ultrasound image enhancement.

Quay further states that fluorocarbon gases generally have high density, high molecular weights, and exhibit extremely low solubility in aqueous systems. *See* Quay, col. 13, lines 26 to 28. Quay provides an analysis of several such fluorocarbon gases, and specifically identifies certain such gases, including SF_6, C_2F_6, C_4F_8, and C_4F_{10}, as possessing the requisite properties that make them suitable for use in microbubble contrast enhancing media. *See e.g.*, Quay, col. 14, table II.

C. Summary of Schneider

Schneider is directed to microbubble suspensions usable as ultrasound imaging contrast agents. *See* abstract. The microbubbles are air or gas globules, in suspension in a liquid, bounded only by an evanescent envelope of the surrounding liquid, which contains surfactants or tensides to control the surface properties thereof. *See* Schneider, page 1, ¶ 3. Suitable surfactants include film forming phospholipids in lamellar or laminar form at the

gas/liquid interface. *See* Schneider, page 10, ¶ 2, and page 22, Claim 1. Schneider also teaches forming microbubble precursors under an atmosphere of one gas, such as air, drying the precursors under reduced pressure, and restoring the pressure with a second gas, to create microbubble preparations containing said second gas. *See* Schneider, page 9, ¶ 2. Schneider does not, however, disclose the physiologically acceptable gases claimed in the reissue application.

D. Summary of Bichon

Bichon is directed to ultrasound contrast agents comprising air or gas filled microballoons bounded by an interfacially deposited polymer membrane. *See* abstract. Suitable polymers include polylactic or polyglycolic acid and their copolymers, proteins such as albumin, and derivatives of polyglutamic and polyaspartic acids. *See* Bichon, col. 9, lines 2 to 40. The microballoons are formed in the presence of air or a selected gas. *See* Bichon, col. 6, lines 35 to 40. As with Schneider, Bichon does not disclose the physiologically acceptable gases claimed in the reissue application.

E. Applicants' Claims are Unpatentable.

From the foregoing, it is abundantly clear that all of the limitations of the instant claims are present in the combined teachings in Quay, Schneider and Bichon. In this connection, Schneider and Bichon describe methods of making microbubble and microballoon ultrasound contrast agents that are of the general type claimed by Applicants. In fact, it is stated directly in the '774 patent that Bichon describes the preferred microballoons of the present application, and Schneider describes the preferred microbubbles. *See* '774 patent, col. 4, lines 33 to 36. The only element of the instant claims lacking from the disclosures of these applications is an identification of the particular gases that Applicants have selected.

Quay, a reference in the same specific field as Schneider and Bichon, describes both the selection criteria and several of the particular gases instantly claimed. Applicants state that suitable gases are those in which the solubility of the gas, divided by the square root of the molecular weight of the gas, is less than or equal to .0031. *See* '774 patent, col. 4, line 5. In

other words, Applicants select gases that have low solubility and high molecular weight, ***which are exactly the same criteria taught by Quay***. Moreover, Quay has specifically identified SF_6, C_2F_6, C_4F_8, and C_4F_{10}, all of which are instantly claimed, as being gases that are suitable for use in stable, long-lived ultrasound contrast media.

It is therefore evident that the combined teachings of Quay and Schneider describe methods for making microbubble contrast media as instantly claimed. Similarly, the combination of Quay with Bichon describes the methods of making microballoon contrast agents defined by Applicants' claims. The motivation to combine these references comes from the references themselves, as each is directed to the art of the ultrasound contrast agents, and methods for their preparation. None of the pending claims of the instant application are patentable over the combined teachings of these references.

CONCLUSION

For the foregoing reasons, Protestor submits that all of the claims in the Reissue Application are unpatentable under 35 U.S.C. § 103 over Quay, in view of Schneider and/or Bichon. Accordingly, Protestor submits that none of the claims in the Reissue Application should be allowed.

Respectfully submitted,

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Date: May 30, 2001

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re **REISSUE APPLICATION** of:

Michel Schneider, et al.

Serial No.: **09/115,963** Group Art Unit: **1616**

Filed: **July 15, 1998** Examiner: **G. Hollinden**

(Reissue Application of U.S. Patent No. 5,413,774, Granted May 9, 1995)

For: **Long Lasting Aqueous Dispersions of Suspensions of Pressure-Resistant Gas-Filled Microvesicles and Methods for the Preparation Thereof**

Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATE OF SERVICE

Sir:

I hereby certify that on May 30, 2001, each of the following documents:

1. **Transmittal Letter;**
2. **Protest Under 37 C.F.R. §1.291;**
3. **Exhibit 1**: U.S. Patent No. 5,393,524 to Quay;
4. **Exhibit 2**: WO 91/15244 to Schneider et al; and
5. **Exhibit 3**: EP 0 458 745 A1 to Bichon et al.

are being served on Reissue Applicants' Attorney by being deposited with the U.S. Postal Service, first class postage prepaid, in an envelope addressed to Reissue Applicants' Attorney:

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US005393524A

United States Patent [19]

Quay

[11] Patent Number: 5,393,524

[45] Date of Patent: Feb. 28, 1995

[54] METHODS FOR SELECTING AND USING GASES AS ULTRASOUND CONTRAST MEDIA

[75] Inventor: Steven C. Quay, Los Angeles, Calif.

[73] Assignee: Sonus Pharmaceuticals Inc., Costa Mesa, Calif.

[21] Appl. No.: 71,377

[22] Filed: Jun. 4, 1993

Related U.S. Application Data

[63] Continuation of Ser. No. 761,311, Sep. 17, 1991, abandoned.

[51] Int. Cl.[6] A61K 49/02

[52] U.S. Cl. 424/9; 424/673; 128/662.02; 128/661.02; 128/660.01; 514/744; 514/746; 514/752; 514/754; 514/757

[58] Field of Search 424/9, 673; 128/662.02, 128/661.02, 660.01; 514/754, 746, 744, 757, 752

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(List continued on next page.)

Primary Examiner—Shailendra Kumar
Attorney, Agent, or Firm—Limbach & Limbach

[57] ABSTRACT

Disclosed herein are agents for enhancing the contrast in an ultrasound image. These agents are extremely small bubbles, or "microbubbles," comprised of specially selected gases. The microbubbles described herein exhibit long life spans in solution and may be produced at a size small enough to traverse the lungs, thus enabling improved ultrasound imaging of the cardiovascular system and other vital organs. Also disclosed herein is a method for selecting gases from which contrast agents may be produced. The method is based on calculations using inherent physical properties of gases and describes a means to associate the properties of a gas with the time for dissolution of a microbubble comprised of the gas.

13 Claims, No Drawings

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METH DS FOR SELECTING AND USING GASES AS ULTRASOUND CONTRAST MEDIA

This is a continuation of co-pending application Ser. No. 07/761,311, filed on Sep. 09, 1991, now abandoned.

DESCRIPTION

This invention relates to agents that enhance the contrast in an ultrasound image generated for use in medical diagnosis. The contrast-enhancing media disclosed herein are comprised of extremely small gas bubbles which are present in a solution that is infused into the body during or just before an ultrasound image is generated. This invention is also directed to a method for enhancing such images by selecting gases from which a collection of free gas microbubbles can be prepared that have novel and superior properties. These microbubbles, composed of the gases whose selection is enabled by the process of this invention, may be extremely small in size and yet survive in the bloodstream long enough to allow contrast-enhanced imaging of parts of the cardiovascular system, peripheral vascular system, and vital organs previously believed to be inaccessible to free gas microbubbles.

BACKGROUND

When using ultrasound to obtain an image of the internal organs and structures of a human or animal, ultrasound waves—waves of sound energy at a frequency above that discernable by the human ear—are reflected as they pass through the body. Different types of body tissue reflect the ultrasound waves differently and the reflections, often aptly described as "echoes," that are produced by the ultrasound waves reflecting off different internal structures are detected and converted electronically into a visual display. This display may prove invaluable to a physician or other diagnostician in several ways, including evaluating the progression of cardiovascular disease or the existence or nature of a tumor.

For some medical conditions, obtaining a useful image of the organ or structure of interest is especially difficult because the details of the structure may not be adequately discernible from the surrounding tissue in an ultrasound image produced by the reflection of ultrasound waves absent a contrast-enhancing agent. Additionally, traditional ultrasound images are notoriously poor in quality and resolution. For these reasons, detection and observation of certain physiological conditions may be substantially improved by enhancing the contrast in an ultrasound image by infusing an agent into an organ or other structure of interest. In other cases, detection of the movement of the contrast-enhancing agent itself is particularly important. For example, a distinct blood flow pattern that is known to result from particular cardiovascular abnormalities may only be discernible by infusing a contrasting agent into the bloodstream and observing the dynamics of the blood flow.

Medical researchers have made extensive investigation into the use of solids, gases and liquids in an attempt to discover ultrasound contrast-enhancing agents suitable for particular diagnostic purposes. Composite substances such as gelatin encapsulated microbubbles, gas-incorporated liposomes, sonicated partially denatured proteins and emulsions containing highly fluorinated organic compounds have also been studied in an attempt to develop an agent that has certain ideal qualities, primarily, stability in the body and the ability to provide significantly enhanced contrast in an ultrasound image.

Small bubbles of a gas, termed "microbubbles," are readily detected in an image produced using standard ultrasound imaging techniques. When infused into the bloodstream or a particular site in the body, microbubbles enhance the contrast between the region containing the microbubbles and the surrounding tissue.

A substantial amount of the research effort directed at contrast-enhancing agents has focused on the use of extremely small gas bubbles. Investigators have long known that free gas bubbles provide a highly effective contrast agent because a gas bubble has unique physical characteristics that affect ultrasound energy as it is directed through the body. The advantages offered by free gas bubbles as opposed to liquid or solid agents that exhibit contrast enhancement is described in detail below in the context of the discussion of ultrasound diagnostic techniques.

Despite the known advantages, however, the rapid dissolution of free gas bubbles in solutions such as blood or many aqueous intravenous solutions, severely limits their use as an ultrasound contrast-enhancing agent. The most important limitations are the size of the microbubble and the length of time that a microbubble will exist before dissolving into the solution.

Examining the size requirements for microbubbles more closely, the gas bubbles must, of course, be sufficiently small that a suspension of the bubbles does not carry the risk of embolism to the organism in which they are infused. At the same time, extremely small free gas bubbles composed of the gases generally used in ultrasound contrast imaging dissolve into solution so rapidly that their image-enhancing capability exists only immediately proximate to the infusion site. An additional obstacle exists for ultrasound imaging of the cardiovascular system. Medical researchers have studied the time required for microbubbles composed of ordinary air, pure nitrogen, pure oxygen, or carbon dioxide, to dissolve into solution. Microbubbles of these gases that are sufficiently small to be able to pass through the lungs and reach the left heart, less than about 8 microns in diameter, have a life span of less than approximately 0.25 seconds. Meltzer, R. S., Tickner, E. G., Popp, R. L., "Why Do the Lungs Clear Ultrasonic Contrast?" *Ultrasound in Medicine and Biology*, Vol. 6, p.263, 267 (1980). Since it takes over 2 seconds for blood to pass through the lungs, microbubbles of these gases would be fully dissolved during passage through the lungs and would never reach the left heart. Ibid. Primarily because of this tradeoff between bubble size and life span, many researchers concluded that free gas microbubbles were not useful as a contract-enhancing agent for ultrasound diagnosis of certain parts of the cardiovascular system.

However, the ultrasound contrast-enhancing media described herein comprises microbubbles, composed of the gases whose selection is also provided by this invention, are sufficiently small that they pass through the pulmonary capillary diameter of approximately 8 microns and thereby allow contrast-enhanced ultrasound diagnosis of the left chambers of the heart. The free gas microbubbles survive in the bloodstream long enough that they may be peripherally intravenously infused, travel through the right heart, through the lungs, and into the left cardiac chambers without dissolving into

solution. Additionally, certain of these media have extremely long persistence in solution and will enable contrast-enhancement of many other organs and structures.

This invention overcomes many of the inherent limitations thought to exist with the use of free gas microbubbles by providing, in part, a method for selecting special gases based on particular physical criteria such that microbubbles composed of these gases do not suffer from the same limitations as the microbubbles previously investigated. Therefore, it has been discovered that the ultrasound contrast-enhancing media described herein comprising a composition of microbubbles produced using a gas or combination of gases selected by the physical and chemical parameters disclosed herein can exist for a sufficient length of time and be of sufficiently small size that their stability in the bloodstream allows enhanced ultrasound contrast imaging of particular structures in the body previously thought inaccessible to free gas microbubbles.

Techniques For Measuring Ultrasound Contrast-Enhancement Phenomena

To more fully appreciate the subject matter of the present invention, it is useful to describe what is presently known about the technology of ultrasound imaging and to review the search for improved ultrasound contrast-enhancing agents in that light.

Materials that are useful as ultrasound contrast agents operate by having an effect on ultrasound waves as they pass through the body and are reflected to create the image from which a medical diagnosis is made. In an attempt to develop an efficient image-contrast agent, one skilled in the art recognizes that different types of substances affect ultrasound waves in different ways and to varying degrees. Moreover, certain of the effects caused by contrast-enhancing agents are more readily measured and observed than others. Thus, in selecting an ideal composition for a contrast-enhancing agent, one would prefer the substance that has the most dramatic effect on the ultrasound wave as it passes through the body. Also, the effect on the ultrasound wave should be easily measured. There are three main contrast-enhancing effects which can be seen in an ultrasound image: backscatter, beam attenuation, and speed of sound differential. Each of these effects will be described in turn.

A. BACKSCATTER

When an ultrasound wave that is passing through the body encounters a structure, such as an organ or other body tissue, the structure reflects a portion of the ultrasound wave. Different structures within the body reflect ultrasound energy in different ways and in varying strengths. This reflected energy is detected and used to generate an image of the structures through which the ultrasound wave has passed. The term "backscatter" refers to the phenomena in which ultrasound energy is scattered back towards the source by a substance with certain physical properties.

It has long been recognized that the contrast observed in an ultrasound image may be enhanced by the presence of substances known to cause a large amount of backscatter. When such a substance is administered to a distinct part of the body, the contrast between the ultrasound image of this part of the body and the surrounding tissues not containing the substance is enhanced. It is well understood that, due to their physical properties, different substances cause backscatter in varying degrees. Accordingly, the search for contrast-enhancing agents has focused on substances that are stable and non-toxic and that exhibit the maximum amount of backscatter.

Making certain assumptions about the way a substance reflects ultrasound energy, mathematical formulae have been developed that describe the backscatter phenomenon. Working with these formulae, a skilled researcher can estimate the ability of gas, liquid, and solid contrast-enhancing agents to cause backscatter and the degree to which a particular substance causes measurable backscatter can be compared with other substances based on the physical characteristics known to cause the backscatter phenomenon. As a simple example, the ability of substance A to cause backscatter will be greater than substance B, if, all other factors being equal, substance A is larger than substance B. Thus, when both substances are encountered by an ultrasound wave, the larger substance scatters a greater amount of the ultrasound wave.

The capability of a substance to cause backscatter of ultrasound energy also depends on other characteristics of the substance such as its ability to be compressed. Of particular importance is the dramatic increase in backscatter caused by gas bubbles due to the bubble resonance phenomenon which is described below. When examining different substances, it is useful to compare one particular measure of the ability of a substance to cause backscatter known as the "scattering cross-section."

The scattering cross-section of a particular substance is proportional to the radius of the scatterer, and also depends on the wavelength of the ultrasound energy and on other physical properties of the substance. J. Ophir and K. J. Parker, *Contrast Agents in Diagnostic Ultrasound*, Ultrasound in Medicine & Biology, Vol. 15, n. 4, p. 319, 323 (1989).

The scattering cross-section of a small scatterer, σ, can be determined by a known equation:

$$\sigma = \left[\frac{4}{9}\pi a^2 (ka)^4\right]\left[\left|\frac{\kappa_s - \kappa}{\kappa}\right|^2 + \frac{1}{3}\left|\frac{3(\rho_s - \rho)}{2\rho_s - \rho}\right|^2\right]$$

where $k = 2\pi/\lambda$, where λ is the wavelength; a = the radius of the scatterer; κ_s = adiabatic compressibility of the scatterer; κ = adiabatic compressibility of the medium in which the scatterer exists, ρ_s = density of the scatterers and ρ = the density of the medium in which the scatterer exists. P. M. Morse and K. U. Ingard, *Theoretical Acoustics*, p. 427, McGraw Hill, New York (1968).

In evaluating the utility of different substances as image contrasting agents, one can use this equation to determine which agents will have the higher scattering cross-section and, accordingly, which agents will provide the greatest contrast in an ultrasound image.

Referring to the above equation, the first bracketed quantity in the above equation can be assumed to be constant for the purpose of comparing solid, liquid and gaseous scatterers. It can be assumed that the compressibility of a solid particle is much less than that of the surrounding medium and that the density of the particle is much greater. Using this assumption, the scattering cross section of a solid particle contrast-enhancing

agent has been estimated as 1.75. Ophir and Parker, supra, at 325.

For a pure liquid scatterer, the adiabatic compressibility and density of the scatterer ρ_s and the surrounding medium ρ are likely to be approximately equal which would, from the above equation, yield the result that liquids would have a scattering cross-section of zero. However, liquids may exhibit some backscatter if large volumes of a liquid agent are present presumably because the term α in the first bracketed quantity in the above equation may become sufficiently large. For example, if a liquid agent passes from a very small vessel to a very large one such that the liquid occupies substantially all of the vessel the liquid may exhibit measurable backscatter. Nevertheless, in light of the above equation and the following, it is appreciated by those skilled in the art that pure liquids are relatively inefficient scatterers compared to free gas microbubbles.

It is known that changes in the acoustic properties of a substance are pronounced at the interface between two phases, i.e. liquid/gas, because the reflection characteristics of an ultrasound wave change markedly at this interface. Additionally, the scatter cross-section of a gas is substantially different than a liquid or solid, in part, because a gas bubble can be compressed to a much greater degree than a liquid or solid. The physical characteristics of gas bubbles in solution are known and standard values for compressibility and density figures for ordinary air can be used in the above equation. Using these standard values, the result for the second bracketed term alone in the above equation is approximately 10^{14}, Ophir and Parker supra, at 325, with the total scattering cross section varying as the radius α of the bubble varies. Moreover, free gas bubbles in a liquid exhibit oscillatory motion such that, at certain frequencies, gas bubbles will resonate at a frequency near that of the ultrasound waves commonly used in medical imaging. As a result, the scattering cross-section of a gas bubble can be over a thousand times larger than its physical size.

Therefore, it is recognized that gas micro-bubbles are superior scatterers of ultrasound energy and would be an ideal contrast-enhancing agent if the obstacle of their rapid dissolution into solution could be overcome.

B. BEAM ATTENUATION

Another effect which can be observed from the presence of certain solid contrast-enhancing agents, is the attenuation of the ultrasound wave. Image contrast has been observed in conventional imaging due to localized attenuation differences between certain tissue types. K. J. Parker and R. C. Wang, "Measurement of Ultrasonic Attenuation Within Regions Selected from B-Scan Images," *IEEE Trans. Biomed. Engr. BME* 30(8), p. 431–37 (1983); K. J. Parker, R. C. Wang, and R. M. Lerner, "Attenuation of Ultrasound Magnitude and Frequency Dependence for Tissue Characterization," Radiology, 153(3), p. 785–88 (1984). It has been hypothesized that measurements of the attenuation of a region of tissue taken before and after infusion of an agent may yield an enhanced image. However, techniques based on attenuation contrast as a means to measure the contrast enhancement of a liquid agent are not well-developed and, even if fully developed, may suffer from limitations as to the internal organs or structures with which this technique can be used. For example, it is unlikely that a loss of attenuation due to liquid contrast agents could be observed in the image of the cardiovascular system because of the high volume of liquid contrast agent that would need to be present in a given vessel before a substantial difference in attenuation could be measured.

Measurement of the attenuation contrast caused by microspheres of Albunex (Molecular Biosystems, San Diego, Calif.) in vitro has been accomplished and it has been suggested that in vivo attenuation contrast measurement could be achieved. H. Bleeker, K. Shung, J. Burnhart, "On the Application of Ultrasonic Contrast Agents for Blood Flowometry and Assessment of Cardiac Perfusion," *J. Ultrasound Med.* 9:461–471 (1990). Albunex is a suspension of 2–4 micron encapsulated air-filled microspheres that have been observed to have acceptable stability in vivo and are sufficiently small in size that contrast enhancement can occur in the left atrium or ventricle. Also, attenuation contrast resulting from iodipamide ethyl ester (IDE) particles accumulated in the liver has been observed. Under such circumstances, the contrast enhancement is believed to result from attenuation of the ultrasound wave resulting from the presence of dense particles in a soft medium. The absorption of energy by the particles occurs by a mechanism referred to as "relative motion." The change in attenuation caused by relative motion can be shown to increase linearly with particle concentration and as the square of the density difference between the particles and the surrounding medium. K. J. Parker, et al., "A Particulate Contrast Agent with Potential for Ultrasound Imaging of Liver," *Ultrasound in Medicine & Biology*, Vol. 13, No. 9, p. 555, 561 (1987). Therefore, where substantial accumulation of solid particles occurs, attenuation contrast may be a viable mechanism for observing image contrast enhancement although the effect is of much smaller magnitude than the backscatter phenomenon and would appear to be of little use in cardiovascular diagnoses.

C. SPEED OF SOUND DIFFERENTIAL

An additional possible technique to enhance contrast in an ultrasound image has been proposed based on the fact that the speed of sound varies depending on the media through which it travels. Therefore, if a large enough volume of an agent, through which the speed of sound is different than the surrounding tissue, can be infused into a target area, the difference in the speed of sound through the target area may be measurable. Presently, this technique is only experimental.

Therefore, considering the three techniques described above for contrast enhancement in an ultrasound image, the marked increase in backscatter caused by free gas microbubbles is the most dramatic effect and contrast-enhancing agents that take advantage of this phenomenon would be the most desirable if the obstacle of their limited stability in solution could be overcome.

The Materials Presently Used as Contrast-Enhancing Agents

In light of what is known about the various techniques described above, attempts to develop a contrast-enhancing agent whose presence generates substantial contrast in an ultrasound image, and whose survival in vivo is sufficiently long to allow contrast-enhanced imaging of the cardiovascular system, has led to the investigation of a broad variety of substances—gases, liquids, solids, and combinations of these—as potential contrast-enhancing agents.

A. SOLID PARTICLES

Typically, the solid substances that have been studied as potential contrast-enhancing agents are extremely small particles that are manufactured in uniform size. Large numbers of these particles can be infused and circulate freely in the bloodstream or they may be injected into a particular structure or region in the body.

IDE particles are solid particles that can be produced in large quantities with a relatively narrow size distribution of approximately 0.5–2.0 microns. Sterile saline injections of these particles may be injected and will tend to accumulate in the liver. Once a substantial accumulation occurs, contrast enhancement may be exhibited by either attenuation contrast or backscatter mechanisms. Although suspensions comprising these solid particles dispersed in a liquid may exhibit acceptable stability, the backscatter or attenuation effects are relatively minor compared to free gas bubbles and a substantial accumulation of the particles must occur before appreciable contrast is observed in an ultrasound image. Thus, use of these suspensions has been limited to certain cell types in which the particles have the tendency to coagulate because unless the suspension becomes highly concentrated in particular tissue, the contrast enhancement will be minor.

SHU-454 (Schering, A. G., West Berlin, Germany) is an experimental contrast-enhancing agent in powder form that, when mixed with a saccharide diluent, forms a suspension of crystals of various rhomboid and polyhedral shapes ranging in size from 5 to 10 microns. Although the precise mechanism by which these crystals enhance ultrasound contrast is not completely understood, it is suspected that the crystals may trap microbubbles in their structure or that the crystals themselves may backscatter ultrasound energy by an as-yet undetermined mechanism.

B. LIQUIDS AND EMULSIONS

In another attempt to achieve a satisfactory agent, emulsions are prepared by combining a chemical species compatible with body tissue and a species that provides high ultrasound contrast enhancement. European Patent Application 0231091 discloses emulsions of oil in water containing highly fluorinated organic compounds that have been studied in connection with their possible use as a blood substitute and are also capable of providing enhanced contrast in an ultrasound image.

Emulsions containing perfluorooctyl bromide (PFOB) have also been examined. Perfluorooctyl bromide emulsions are liquid compounds known to have the ability to transport oxygen. PFOB emulsions have exhibited a limited utility as ultrasound contrast agents because of a tendency to accumulate in certain types of cells. Although the mechanism is not completely understood, PFOB emulsions may provide ultrasound contrast because of their high density and relatively large compressibility constant.

U.S. Pat. No. 4,900,540 describes the use of phospholipid-based liposomes containing a gas or gas precursor as a contrast-enhancing agent. A liposome is a microscopic, spherical vesicle, containing a bilayer of phospholipids and other amphipathic molecules and an inner aqueous cavity, all of which is compatible with the cells of the body. In most applications, liposomes are used as a means to encapsulate biologically active materials. The above reference discloses the use of a gas or gas precursors incorporated into the liposome core to provide a longer life span for the gas when infused into th body. Production f stable liposomes is an expensive and time consuming process requiring specialized raw materials and equipment.

C. MICROBUBBLES

As noted above, a critical parameter that must be satisfied by a microbubble used as a contrast-enhancing agent is size. Free gas microbubbles larger than approximately 8 microns may still be small enough to avoid impeding blood flow or occluding vascular beds. However, microbubbles larger than 8 microns are removed from the bloodstream when blood flows through the lungs. As noted above, medical researchers have reported in the medical literature that microbubbles small enough to pass through the lungs will dissolve so quickly that contrast enhancement of left heart images is not possible with a free gas microbubble. Meltzer, R. S., Tickner, E. G., Popp, R. L., "Why Do the Lungs Clear Ultrasonic Contrast?" *Ultrasound in Medicine and Biology*, Vol. 6, p.263, 267 (1980).

However, cognizant of the advantages to be gained by use of microbubbles as contrast-enhancing agents by virtue of their large scattering cross-section, considerable attention has been focused on developing mixtures containing microbubbles that are rendered stable in solution. Enhancing the stability of gas microbubbles may be accomplished by a number of techniques.

Each of the following techniques essentially involves suspending a collection of microbubbles in a substrate in which a bubble of ordinary gas is more stable than in the bloodstream.

In one approach, microbubbles are created in viscous liquids that are injected or infused into the body while the ultrasound diagnosis is in progress. The theory behind the use of viscous fluids involves an attempt to reduce the rate at which the gas dissolves into the liquid and, in so doing, provide a more stable chemical environment for the bubbles so that their lifetime is extended.

Several variations on this general approach have been described. EPO Application No. 0324938 describes a viscous solution of a biocompatible material, such as a human protein, in which microbubbles are contained. By submitting a viscous protein solution to sonication, microbubbles are formed in the solution. Partial denaturation of the protein by chemical treatment or heat provides additional stability to microbubbles in the solution by decreasing the surface tension between bubble and solution.

Therefore, the above approaches may be classified as an attempt to enhance the stability of microbubbles by use of a stabilizing medium in which the microbubbles are contained. However, none of these approaches have addressed the primary physical and chemical properties of gases which have seriously limited the use of free gas microbubbles in ultrasound diagnosis, particularly with respect to the cardiovascular system. None of these approaches suggest that selection of the gases, by precise criteria, would yield the ability to produce stable microbubbles at a size that would allow transpulmonary contrast-enhanced ultrasound imaging.

The behavior of microbubbles in solution can be described mathematically based on certain parameters and characteristics of the gas of which the bubble is formed and the solution in which the bubble is present. Depending on the degree to which a solution is saturated with the gas of which the microbubbles are

formed, the survival time of the microbubbles can be calculated. P. S. Epstein, M. S. Plesset, "On the Stability of Gas Bubbles in Liquid-Gas Solutions," *The Journal of Chemical Physics*, Vol 18, n. 11, 1505 (1950). Based on calculations, it is apparent that as the size of the bubble decreases, the surface tension between bubble and surrounding solution increases. As the surface tension increases, the rate at which the bubble dissolves into the solution increases rapidly and, therefore, the size of the bubble decreases more and more rapidly. Thus, the rate at which the bubble shrinks increases as the size of the bubble decreases. The ultimate effect of this is that a population of small free gas microbubbles composed of ordinary air dissolves so rapidly that the contrast-enhancing effect is extremely short lived. Using known mathematical formula, one can calculate that a microbubble of air that is 8 microns in diameter, which is small enough to pass through the lungs, will dissolve in between 190 and 550 milliseconds depending on the degree of saturation of the surrounding solution. Based on these calculations, medical investigators studying the way in which the lungs remove ultrasound contrast agent have calculated the dissolution times of oxygen and nitrogen gas microbubbles in human and canine blood and have concluded that free gas microbubble contrast agents will not allow contrast-enhanced imaging of the left ventricle because of the extremely brief life of the microbubbles.

The physical properties of systems that feature gas bubbles or gases dissolved in liquid solutions have been investigated in detail including the diffusion of air bubbles formed in the cavitating flow of a liquid and the scatter of light and sound in water by gas bubbles.

The stability of gas bubbles in liquid-gas solution has been investigated both theoretically, Epstein P. S. and Plesset M. S., On the Stability of Gas Bubbles in Liquid-gas Solutions, J. Chem. Phys. 18:1505–1509 (1950) and experimentally, Yang W. J., Dynamics of Gas Bubbles in Whole Blood and Plasma, J. Biomech 4:119–125 (1971); Yang W. J., Echigo R., Wotton D. R., and Hwang J. B., Experimental Studies of the Dissolution of Gas Bubbles in Whole Blood and Plasma-I. Stationary Bubbles. J. Biomech 3:275–281 (1971); Yang W. J., Echigo R., Wotton D. R., Hwang J. B., Experimental Studies of the Dissolution of Gas Bubbles in Whole Blood and Plasma-II. Moving Bubbles or Liquids. J. Biomech 4:283–288 (1971). The physical and chemical properties of the liquid and the gas determine the kinetic and thermodynamic behavior of the system. The chemical properties of the system which influence the stability of a bubble, and accordingly the life time, are the rate and extent of reactions which either consume, transform, or generate gas molecules.

For example, a well known reaction that is observed between a gas and a liquid takes place when carbon dioxide gas is present in water. As the gas dissolves into the aqueous solution, carbonic acid is created by hydration of the carbon dioxide gas. Because carbon dioxide gas is highly soluble in water, the gas diffuses rapidly into the solution and the bubble size diminishes rapidly. The presence of the carbonic acid in the solution alters the acid-base chemistry of the aqueous solution and, as the chemical properties of the solution are changed by dissolution of the gas, the stability of the carbon dioxide gas bubbles changes as the solution becomes saturated. In this system, the rate of dissolution of a gas bubble depends in part on the concentration of carbon dioxide gas that is already dissolved in solution.

However, depending on the particular gas or liquid present in the system, the gas may be substantially insoluble in the liquid and dissolution of a gas bubble will be slower. In this situation, it has been discovered that it is possible to calculate bubble stability in a gas-liquid system by examining certain physical parameters of the gas.

BRIEF DESCRIPTION OF THE INVENTION

It has been discovered that it is possible to identify chemical systems where extremely small gas bubbles are not reactive in an aqueous solution. Relying on the method disclosed herein one skilled in the art may specially select particular gases based on their physical and chemical properties for use in ultrasound imaging. These gases can be used to produce the contrast-enhancing media that is also the subject matter of this invention. The microbubbles can be produced using certain existing techniques that use ordinary air, and can be infused as in a conventional ultrasound diagnosis.

The method that is the subject matter of this invention requires that calculations be made, consistent with the equations provided herein, based on the intrinsic physical properties of a gas and a liquid. Particularly, the density of a gas, the solubility of a gas in solution, and the diffusivity of a gas in solution, which in turn is dependent on the molar volume of the gas and the viscosity of the solution, are used in the equations disclosed below. Thus, by the method disclosed herein, the physical properties of a given gas-liquid system can be evaluated, the rate and extent of bubble collapse can be estimated, and gases that would constitute effective contrast-enhancing agents can be selected based on these calculations. Using existing techniques, substantially improved contrast-enhancing media may then be produced and used to improve the quality and usefulness of ultrasound imaging.

DETAILED DESCRIPTION OF THE INVENTION

To understand the method of this invention, it is useful to derive the mathematical relationships that describe the parameters of a gas-liquid system and the effect on bubble stability that occurs when a value for one or more of these parameters is altered. It is assumed that, at an initial time, To, a spherical gas bubble of gas X, with a radius of R_o, is placed in a solution in which the initial concentration of gas X dissolved in the solution is equal to zero. Over some period of time, the bubble of gas X will dissolve into the solvent at which time its radius R will equal zero. Assume further that the solution is at constant temperature and pressure and that the dissolved gas concentration for a solution saturated with the particular gas is designated C_s. Thus, at T_o the concentration of the gas in the solution is zero, meaning that none of the gas has yet dissolved and all of the gas that is present is still contained within the bubble of radius R_o.

As time progresses, due to the difference in the concentration of the gas in the bubble and the gas in solution, the bubble will tend to shrink as gas in the bubble is dissolved into the liquid by the process of diffusion. The change in bubble radius from its original radius of R_o to, after the passage of a particular amount of time t, a smaller radius R is expressed by Equation (1),

$$\frac{R}{R_0} = \left[1 - \left(\frac{2DC_s}{\rho R_0^2}\right)t\right]^{\frac{1}{2}}$$

where R is the bubble radius at time t, D is the coefficient of diffusivity of the particular gas in the liquid, and ρ is the density of the particular gas of which the bubble is composed.

It follows that the time T required for a bubble to dissolve completely may be determined from Equation (1) by setting $R/R_0=0$, and solving for T:

$$T = \frac{R_0^2\rho}{2DC_s} \quad \text{Equation (2)}$$

This result qualitatively indicates that bubble stability, and hence life span, is enhanced by increasing the initial bubble size R_0 or by selecting a gas of higher density ρ, lower solubility C_s in the liquid phase, or a lower coefficient of diffusivity D.

The diffusivity D of a gas in a liquid is dependent on the molar volume of the gas (Vm), and the viscosity of the solution (η) as expressed by a known Equation;

$$D = 13.26\times10^{-5}\cdot\eta^{-1.14}\cdot V_m^{-0.589} \quad \text{(Equation 3)}$$

By substituting the expression for D given in Equation (3) into Equation (2), it is revealed that bubble stability is enhanced by using gases of larger molar volume Vm, which tend to have a higher molecular weight, and liquids of higher viscosity.

By way of example, a comparison of the stability of air microbubbles and microbubbles composed of gases specially selected by the method disclosed herein may be made. Taking the value of D for air in water at 22° C. as 2×10^{-5} cm^{2}sec^{-1} and the ratio $C_s/\rho=0.02$ (Epstein and Plesset, Ibid.), one obtains the following data for the time t for complete solution of air bubbles in water (unsaturated with air):

TABLE I

INITIAL BUBBLE DIAMETER, microns	TIME, milliseconds
12	450
10	313
8	200
6	113
5	78
4	50
3	28
2	13
1	3

If the blood transit time from the pulmonary capillaries to the left ventricle is two seconds or more (Hamilton, W. F. editor, Handbook of Physiology, Vol. 2, section 2, CIRCULATION. American Physiology Society, Washington, D.C., p. 709, (1963)), and recognizing that only microbubbles of approximately 8 microns or less will be small enough to pass through the lungs, it is clear that none of these bubbles have a life span in solution long enough to be useful contrast agents for ultrasound contrast-enhanced imaging of the left ventricle.

The method of the present invention allows identification of potentially useful gases by comparing the properties of any particular gas, denoted gas X in the following description, to air. Taking Equations (2) and (3) above, a coefficient Q may be formulated for a particular gas X that will describe the stability of microbubbles composed of gas X in a given liquid. The value f the Q coefficient determined by this method for a particular gas X also can be used to determine the utility of gas X as an ultrasound contrast-enhancing agent as compared to ordinary air.

From Equation (2) above, an equation that describes the time for complete dissolution of a bubble of gas X compared to the same size bubble of ordinary air under identical conditions of solution temperature and solution viscosity may be written based on the physical properties of gas X and air:

$$T_x = T_{air}\left[\frac{\rho_x}{\rho_{air}}\right]\left[\frac{C_s^{air}}{C_s^x}\right]\left[\frac{D_{air}}{13.26\times10^{-5}\cdot\eta^{-1.14}\cdot V_m^{-.589}}\right] \quad \text{Equation (4)}$$

or, if D is known for gas X,

$$T_x = T_{air}\left[\frac{\rho_x}{\rho_{air}}\right]\left[\frac{C_s^{air}}{C_s^x}\right]\left[\frac{D_{air}}{D_x}\right] \quad \text{Equation (5)}$$

To formulate this equation so that the value Q may be obtained to enable comparison of gas X with air, the above equation may be rewritten:

$$T_x = QT_{air} \text{ where} \quad \text{Equation (6)}$$

$$Q = \left[\frac{\rho_x}{\rho_{air}}\right]\left[\frac{C_s^{air}}{C_s^x}\right]\left[\frac{D_{air}}{D_x}\right]$$

Assuming for comparison, a solution of water at 22 degrees C., the density, diffusivity, and solubility of air in the solution are known quantities which may be substituted into the above equation yielding:

$$Q = 4.0\times10^{-7}\left[\frac{\rho_x}{C_s^x D_x}\right] \quad \text{Equation (7)}$$

Substituting Equation (3) into the above for gases whose diffusivity Dx is not readily known, and assuming that the viscosity term η below for water at 22 degrees C. is approximately equal to 1.0 cP,

$$Q = 3.0\times10^{-3}\left[\frac{\rho_x}{C_s^x V_m^{x\,-.589}}\right] \quad \text{Equation (8)}$$

Thus, knowing the density, solubility and molar volume of a gas, this method allows the calculation of the value of the Q coefficient.

If Q is less than one, microbubbles of gas X will be less stable in a given solvent than microbubbles of air. If Q is greater than one, microbubbles formed of gas X are more stable than microbubbles of air and will survive in solution longer than air bubbles. All other properties being the same for a given microbubble size, the time for complete dissolution of a microbubble of gas X is equal to the time for complete dissolution of a microbubble of ordinary air multiplied by the Q coefficient. For example, if the Q coefficient for gas X is 10,000, a microbubble of gas X will survive 10,000 times as long

in solution compared to a microbubble of air. A Q value can be determined for any gas in any solution assuming the quantities identified herein are known or can be estimated.

Different methods for determining or estimating values for the individual parameters of density, diffusivity, and solubility may be needed depending on the chemical structure of the gas. Values for these parameters may or may not be available from known scientific literature sources such as the *Gas Encyclopaedia* or the tabulations published by the American Chemical Society. Values for the density of most gases are readily available from sources such as the *Handbook of Chemistry and Physics*, CRC Press, 72d Ed. (1991-92). Additionally, the solubility in water and molar volume of some gases has been measured with accuracy. In many cases however, calculations for the numerical values for molar volume and solubility may need to be calculated or estimated to provide the data used to determine the value of the Q coefficient for an individual gas by the method described above. An example of the calculation of Q values for a preferred selection of fluorocarbon gases illustrates how the method of this invention can be applied to individual gases.

EXAMPLE

Generally, fluorocarbon gases exhibit extremely low solubility in water, and have high molecular weights, high molar volumes, and high densities. To determine the Q value for several fluorocarbon gases, the solubility, molar volume and density of the individual gases are determined and the values are substituted into Equations (7) or (8) above.

Determination of Gas Solubility for Fluorocarbons

This method for estimating the gas solubility of fluorocarbons uses extrapolation of the experimental data of Kabalnov A. S., Makarov K. N., and Scherbakova O. V. "Solubility of Fluorocarbons in Water as a Key Parameter Determining Fluorocarbon Emulsion Stability," *J. Fluor. Chem.* 50, 271-284, (1990). The gas solubility of these fluorocarbons is determined relative to perfluoro-n-pentane which has a water solubility of 4.0×10^{-6} moles per liter. For a homologous series of non-branched fluorocarbons, the gas solubility may be estimated by increasing or reducing this value by a factor of about 8.0 for each increase or reduction in the number of additional $-CF_2-$ groups present in the molecule.

Determination of Molar Volume

The molar volume (Vm) is estimated from the data of Bondi A., "Van der Waals Volumes and Radii," *J Phys. Chem.* 68, 441-451 (1964). The molar volume of a gas can be estimated by identifying the number and type of atoms that make up the molecule of gas in question. By determining the number and type of atoms present in the molecule and how the individual atoms are bound to each other, known values may be applied for the molecular volume of the individual atoms. By considering the contribution of each individual atom and its frequency of occurrence, one may calculate the total molar volume for a particular gas molecule. This calculation is best demonstrated with an example.

It is known that a carbon molecule in an alkane carbon-carbon bond has a molar volume of 3.3 cubic centimeters per mole, a carbon atom in an alkene carbon-carbon bond has a molar volume of 10.0 cubic centimeters per mole, and when multiple fluorine atoms are bound to an alkane carbon, a fluorine atom has a molar volume of 6.0 cubic centimeters per mole.

Examining octafluoropropane, this molecule contains three carbon atoms in alkane carbon-carbon bonds (3 atoms at 3.3 cubic centimeters per mole) and 6 fluorine atoms bound to alkane carbons (6 atoms at 6.0 cubic centimeters per mole), hence, octafluoropropane has a molar density of 58 cubic centimeters per mole.

Once density, molar volume, and solubility are determined, the Q value is calculated using Equation 8 above.

The following Table lists the Q value for several fluorocarbon gases based on the calculations detailed above, the values for carbon dioxide are included for comparison.

TABLE II

GAS	DENSITY kg/m³	SOLUBILITY micromoles/liter	MOLAR VOLUME cm³/mole	Q
Carbon dioxide	1.977	33000	19.7	1
Sulfur Hexafluoride	5.48	220	47	722
Hexafluoropropylene	10(*)	2000	49	188
Octafluoropropane	10.3	260	58	1289
Hexafluoroethane	8.86	2100	43	916
Octafluoro-2-butene	10(*)	220	65	1294
Hexafluoro-2-butyne	9(*)	2000	58	148
Hexafluorobuta-1,3-diene	9(*)	2000	56	145
Octafluorocyclobutane	9.97	220	61	1531
Decafluorobutane	11.21	32	73	13,154

(*)These density values are estimated from the known density of homologous fluorocarbons.

Once the Q value has been determined the utility of an individual gas as an ultrasound contrast-enhancing agent can be analyzed by determining the life span of a collection of microbubbles composed of the gas in question at different sizes, as was done for air in Table 1 above. Taking the value of Q for decafluorobutane and examining the time necessary for various sized bubbles to dissolve in water, one obtains the values in Table III below by multiplying each of the time values in Table I by the Q value for decafluorobutane:

TABLE III

INITIAL BUBBLE DIAMETER, microns	TIME, minutes
12	99
10	69
8	44
6	25
5	17
4	11
3	6.1
2	2.9
1	0.7

Notice that the time scale in Table III is minutes rather than milliseconds as was the case for air. All bubbles of decafluorobutane, even as small as 1 micron, can be injected peripherally and will not dissolve into solution during the approximately 10 seconds needed to reach the left ventricle. Similar calculations can be performed for a gas with any Q coefficient. Based on these calculations, a gas needs a Q value of at least 30 to comprise a useful agent for ultrasound contrast enhancement. Slightly larger bubbles will be able to pass through the lungs and yet survive long enough to permit both examination of myocardial perfusion and dynamic abdominal organ imaging. Moreover, as with many of the gases identified by this method, decafluorobutane features low toxicity at small dosages and would, therefore, offer substantial advantages as a contrast-enhancing agent in conventional ultrasound diagnosis.

It will be appreciated by those skilled in the art that the low solubilities of these microbubbles in solution will require that production of a suspension of these microbubbles be achieved by a method which, suspends a quantity of gas into liquid rather than by a method such as sonication which creates microbubbles from gases dissolved in solution. Manual creation of a microbubble suspension may be accomplished by several methods. U.S. Pat. No. 4,832,941, the disclosure of which is incorporated herein by reference, refers to a method for producing a suspension of microbubbles with a diameter less than seven microns created by spraying a liquid through a quantity of gas using a three-way tap. Although techniques could vary in practice, the three-way tap is a preferred method to manually suspend a quantity of high Q coefficient gas to produce the contrast-enhancing media described herein.

The general techniques for use of a three-way tap device are well known in connection with preparation of the common Freund's adjuvant for immunizing research animals. Typically, a three-way tap is comprised of a pair of syringes, both of which are connected to a chamber. The chamber has outlet from which the suspension may be collected or infused directly.

Techniques for use of the three-way tap may differ from that described in U.S. Pat. No. 4,832,941 because different gases are being used in this procedure. For example, use of one of the high Q coefficient gases disclosed herein may be more efficient if the system is purged of ordinary air or flushed with another gas before the microbubble suspension is produced.

In a preferred embodiment of the present invention, a 40-50% Sorbitol (D-glucitol) solution is mixed with approximately 1-10% by volume of a high Q-coefficient gas with approximately 5% gas being an optimal value. Sorbitol is a commercially available compound that when mixed in an aqueous solution substantially increases the viscosity of the solution. Higher viscosity solutions, as seen in equation 3 above, extend the life of a microbubble in solution. A 40-50% Sorbitol solution is preferred to maintain as a bolus upon injection; that is as intact as possible without exceeding a tolerable injection pressure. To produce the suspension of microbubbles, a quantity of the chosen gas is collected in a syringe. In the same syringe, a volume of the Sorbitol solution may be contained. A quantity of Sorbitol solution is drawn into the other syringe so that the sum of the two volumes yields the proper percentage of gas based on the volume percentage of microbubbles desired. Using the two syringes, each featuring a very small aperture, the liquid is sprayed into the gas atmosphere approximately 25 times or as many times as is necessary to create a suspension of microbubbles whose size distribution is acceptable for the purposes described herein. This technique may be varied slightly, of course, in any manner that achieves the resulting suspension of microbubbles of the desired size in a desired concentration. Microbubble size may be checked either visually or electronically using a Coulter Counter (Coulter Electronics) by a known method, Butler, B. D., "Production of Microbubble for Use as Echo Contrast Agents," *J. Clin. Ultrasound*, V.14 408 (1986).

Although the invention has been described in some respects with reference to specified preferred embodiments thereof, variations and modifications will become apparent to those skilled in the art. It is, therefore, the intention that the following claims not be given a restrictive interpretation but should be viewed to encompass variations and modifications that are derived from the inventive subject matter disclosed.

I claim:

1. In a method comprising ultrasound imaging, the improvement comprising enhancing the contrast in an ultrasound image by selecting for use as an enhancing agent free gas microbubbles of a biocompatible fluorocarbon containing contrast-enhancing chemical,

said chemical as a gas having a Q coefficient greater than 30, where Q is the ratio of the persistence of microbubbles of said gas in an aqueous solution to the persistence of microbubbles of air in said solution.

2. The method of claim 1 wherein the gas is hexafluoropropylene.

3. The method of claim 1 wherein the gas is octafluoropropane.

4. The method of claim 1 wherein the gas is hexafluoroethane.

5. The method of claim 1 wherein the gas is octafluoro-2-butene.

6. The method of claim 1 wherein the gas is hexafluoro-2-butyne.

7. The method of claim 1 wherein the gas is hexafluorobuta-1,3-diene.

8. The method of claim 1 wherein the gas is octafluorocyclobutane.

9. The method of claim 1 wherein the gas is decafluorobutane.

10. The method of claim 1 wherein the microbubbles are present in an aqueous sorbitol solution.

11. The method of claim 1 wherein the microbubbles are present in a solution to which a biocompatible substance has been added to increase the viscosity of the solution.

12. The method of claim 1 wherein the microbubbles are smaller than 8 microns.

13. In a method of ultrasound imaging, the improvement comprising the steps of:

providing a suspension of biocompatible free gas microbubbles of sulfur hexafluoride to an organism to be imaged; and

subjecting said organism to an ultrasound scan.

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(57) Abstract

Gas or air filled microbubble suspensions in aqueous phases usable as imaging contrast agents in ultrasonic echography. They contain laminarized surfactants and, optionally, hydrophilic stabilizers. The laminarized surfactants can be in the form of liposomes. The suspensions are obtained by exposing the laminarized surfactants to air or a gas before or after admixing with an aqueous phase.

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STABLE MICROBUBBLES SUSPENSIONS INJECTABLE INTO LIVING ORGANISMS

The present invention concerns media adapted for injection into living bodies, e.g. for the purpose of ultrasonic echography and, more particularly, injectable liquid compositions comprising microbubbles of air or physiologically acceptable gases as stable dispersions or suspensions in an aqueous liquid carrier. These compositions are mostly usable as contrast agents in ultrasonic echography to image the inside of blood-stream vessels and other cavities of living beings, e.g. human patients and animals. Other uses however are also contemplated as disclosed hereafter.

The invention also comprises dry compositions which, upon admixing with an aqueous carrier liquid, will generate the foregoing sterile suspension of microbubbles thereafter usable as contrast agent for ultrasonic echography and other purposes.

It is well known that microbodies like microspheres or microglobules of air or a gas, e.g. microbubbles or microballoons, suspended in a liquid are exceptionally efficient ultrasound reflectors for echography. In this disclosure the term of "microbubble" specifically designates air or gas globules in suspension in a liquid which generally results from the introduction therein of air or a gas in divided form, the liquid preferably also containing surfactants or tensides to control the surface properties thereof and the stability of the bubbles. More specifically, one may consider that the internal volume of the microbubbles is limited by the gas/liquid interface, or in other words, [redacted] surfactant ideally [redacted] boundary.

In contrast, the term of "microcapsule" or "microballoon" designates preferably air or gas bodies with a material boundary or envelope formed of molecules thicker than that of the liquid of suspension, e.g. a polymer membrane wall. Both microbubbles and

microballoons are useful as ultrasonic contrast agents. For instance injecting into the blood-stream of living bodies suspensions of gas microbubbles or microballoons (in the range of 0.5 to 10 μm) in a carrier liquid will strongly reinforce ultrasonic echography imaging, thus aiding in the visualization of internal organs. Imaging of vessels and internal organs can strongly help in medical diagnosis, for instance for the detection of cardiovascular and other diseases.

The formation of suspensions of microbubbles in an injectable liquid carrier suitable for echography can follow various routes. For instance in DE-A- 3529195 (Max-Planck Gesell.), there is disclosed a technique for generating 0.5-50 μm bubbles in which an aqueous emulsified mixture containing a water soluble polymer, an oil and mineral salts is forced back and forth, together with a small amount of air, from one syringe into another through a small opening. Here, mechanical forces are responsible for the formation of bubbles in the liquid.

M.W. Keller et al. (J. Ultrasound Med. 5 (1986), 439-8) have reported subjecting to ultrasonic cavitation under atmospheric pressure solutions containing high concentrations of solutes such as dextrose, Renografin-76, Iopamidol (an X-ray contrast agent), and the like. There the air is driven into the solution by the energy of cavitation.

Other techniques rely on the shaking of a carrier liquid in which air containing microparticles have been incorporated, said carrier liquid usually containing, as stabilizers, viscosity enhancing agents, e.g. water-soluble polypeptides or carbohydrates and/or surfactants. It is effectively admitted that the stability of the microbubbles against decay or escape to the atmosphere is controlled by the viscosity and surface properties of the carrier liquid. The air or gas in the microparticles can consist of inter-particle or intra-crystalline entrapped gas, as well as surface adsorbed gas, or gas produced by reactions with the carrier liquid, usually aqueous. All this is fully described for instance in EP-A- 52.575 (Ultra Med. Inc.) in which there are used aggregates of 1 - 50 μm particles of carbohydrates (e.g. galactose, maltose, sorbitol, gluconic acid, sucrose, glucos

and the like) in aqueous solutions of glycols or polyglycols, or other water soluble polymers.

Also, in EP-A- 123.235 and 122.624 (Schering, see also EP-A- 320.433) use is made of air trapped in solids. For instance, 122.624 claims a liquid carrier contrast composition for ultrasonic echography containing microparticles of a solid surfactant, the latter being optionally combined with microparticles of a non-surfactant. As explained in this document, the formation of air bubbles in the solution results from the release of the air adsorbed on the surface of the particles, or trapped within the particle lattice, or caught between individual particles, this being so when the particles are agitated with the liquid carrier.

EP-A- 131.540 (Schering) also discloses the preparation of microbubbles suspensions in which a stabilized injectable carrier liquid, e.g. a physiological aqueous solution of salt, or a solution of a sugar like maltose, dextrose, lactose or galactose, without viscosity enhancer, is mixed with microparticles (in the 0.1 to 1 µm range) of the same sugars containing entrapped air. In order that the suspension of bubbles can develop within the liquid carrier, the foregoing documents recommend that both liquid and solid components be violently agitated together under sterile conditions; the agitation of both components together is performed for a few seconds and, once made, the suspension must then be used immediately, i.e. it should be injected within .5 - 10 minutes for echographic measurements; this indicates that the bubbles in the suspensions are not longlived and one practical problem with the use of microbubbles suspensions for injection is lack of stability with time. The present invention fully remedies this drawback.

In US-A- 4,466,442 (Schering), there is disclosed a series of different techniques for producing suspensions of gas microbubbles in a liquid carrier using (a) a solution of a tenside (surfactant) in a carrier liquid (aqueous) and (b) a solution of a viscosity enhancer as stabilizer. For generating the bubbles, the techniques used there include forcing at high velocity a mixture of (a), (b) and air through a small aperture;

injecting (a) into (b) shortly before use together with a physiologically acceptable gas; or adding an acid to (a) and a carbonate to (b), both components being mixed together just before use and the acid reacting with the carbonate to generat CO_2 bubbles; or adding an over-pressurised gas to a mixture of (a) and (b) under storage, said gas being released into microbubbles at the time when the mixture is used for injection.

The tensides used in component (a) of US-A- 4,466,442 comprise lecithins; esters and ethers of fatty acids and fatty alcohols with polyoxyethylene and polyoxyethylated polyols lik sorbitol, glycols and glycerol, cholesterol; and polyoxy-ethylene-polyoxypropylene polymers. The viscosity raising and stabilizing compounds include for instance mono- and polysaccharides (glucose, lactose, sucrose, dextran, sorbitol); polyols, e.g. glycerol, polyglycols; and polypeptides like proteins, gelatin, oxypolygelatin, plasma protein and the like.

In a typical preferred example of this document, equivalent volumes of (a) a 0.5% by weight aqueous solution of Pluronic® F-68 (a polyoxypropylene-polyoxyethylene polymer) and (b) a 10% lactose solution are vigorously shaken together under sterile conditions (closed vials) to provide a suspension of microbubbles ready for use as an ultrasonic contrast agent and lasting for at least 2 minutes. About 50% of the bubbles had a size below 50 μm.

Although the achievements of the prior art have merit, they suffer from several drawbacks which strongly limit their practical use by doctors and hospitals, namely their relatively short life-span (which makes test reproducibility difficult), relative low initial bubble concentration (the number of bubbles rarely exceeds 10^4 - 10^5 bubbles/ml and the count decreases rapidly with time) and poor reproducibility of the initial bubble count from test to test (which also makes comparisons difficult). Also it is admitted that for efficiently imaging certain organs, e.g. the left heart, bubbles smaller than 50 μm, preferably in th range of 0.5-10 μm, ar required; with long r bubbl s, there ar risks f clots and c nsecutiv mb ly.

Furth rm re, th c mpuls ry pr senc f s lid micr parti-

cles or high concentrations of lectrolytes and ther relativ ly inert solutes in th carri r liquid may b undesirable physiologically in some cases. Finally, the suspensions are totally unstable under storage and cannot be marketed as such; hence great skill is required to prepare the microbubbles at the right moment just before use.

Of course there exists stable suspensions of microcapsules, i.e. microballoons with a solid, air-sealed rigid polymeric membrane which perfectly resist for long storage periods in suspension, which have been developed to remedy this shortcoming (see for instance K.J. Widder, EP-A- 324.938); however the properties of microcapsules in which a gas is entrapped inside solid membrane vesicles essentially differ from that of the gas microbubbles of the present invention and belong to a different kind of art; for instance while the gas microbubbles discussed here will simply escape or dissolve in the blood-stream when the stabilizers in the carrier liquid are excreted or metabolized, the solid polymer material forming the walls of the aforementioned micro-balloons must eventually be disposed of by the organism being tested which may impose a serious afterburden upon it. Also capsules with solid, non-elastic membrane may break irreversibly under variations of pressure.

The composition of the present invention, as defined in claim 1, fully remedies the aforementioned pitfalls.

The term "lamellar form" defining the condition of at least a portion of the surfactant or surfactants of the present composition indicates that the surfactants, in strong contrast with the microparticles of the prior art (for instance EP-A-123.235), are in the form of thin films involving one or more molecular layers (in laminate form). Converting film forming surfactants into lamellar form can easily be done for instance by high pressure homogenization or by sonication under acoustical or ultrasonic frequencies. In this connection, it should be pointed out that the existence of liposomes is a well known and useful illustration of cases in which surfactants, more particularly lipids, ar in lam llar f rm.

Lip s m s lutions are aqueous susp nsi ns f micr sc pic

vesicles, generally sph rically shaped, vhich hold substances encapsulated therein. These vesicles are usually formed of one or more concentrically arranged molecular layers (lamellae) of amphipatic compounds, i.e. compounds having a lipophobic hydrophilic moiety and a lipophilic hydrophobic moiety. See for instance "Liposome Methodology", Ed. L.D. Leserman et al, Inserm 136, 2-8 May 1982). Many surfactants or tensides, including lipids, particularly phospholipids, can be laminarized t correspond to this kind of structure. In this invention, one preferably uses the lipids commonly used for making liposomes, for instance the lecithins and other tensides disclosed in mor detail hereafter, but this does in no way preclude the use of other surfactants provided they can be formed into layers or films.

It is important to note that no confusion should be made between the present invention and the disclosure of Ryan (US-A-4,900,540) reporting the use of air or gas filled liposomes for echography. In this method Ryan encapsulates air or a gas within liposomic vesicles; in embodiments of the present invention microbubbles or air or a gas are formed in a suspension of liposomes (i.e. liquid filled liposomes) and the liposomes apparently stabilize the microbubbles. In Ryan, the air is inside the liposomes, which means that within the bounds of the presently used terminology, the air filled liposomes of Ryan belong to the class of microballoons and not to that of the microbubbles of the present invention.

Practically, to achieve the suspensions of microbubbles according to the invention, one may start with liposomes suspensions or solutions prepared by any technique reported in the prior art, with the obvious difference that in the present case the liposomic vesicles are preferably "unloaded", i.e. they do not need to keep encapsulated therein any foreign material other than the liquid of suspension as is normally the object of classic liposomes. Hence, preferably, the liposomes f th pr s nt inv nti n vill c ntain an aque us phase identical or similar t th aqu us phase f the s luti n its lf. Then air or a gas is intr duc d int th lipos me s luti n s that a

suspension f. micr bubbles will f rm, said susp nsi n being stabilized by the presence f th surfactants in lam llar f rm. Notwithstanding, the material making the liposome walls can be modified within the scope of the present invention, for instance by covalently grafting thereon foreign molecules designed for specific purposes as will be explained later.

The preparation of liposome solutions has been abundantly discussed in many publications, e.g. US-A- 4,224,179 and WO-A-88/09165 and all citations mentioned therein. This prior art is used here as reference for exemplifying the various methods suitable for converting film forming tensides into lamellar form. Another basic reference by M.C. Woodle and D. Papahadjopoulos is found in "Methods in Enzymology" 171 (1989), 193.

For instance, in a method disclosed in D.A. Tyrrell et al, Biochimica & Biophysica Acta 457 (1976), 259-302, a mixture of a lipid and an aqueous liquid carrier is subjected to violent agitation and thereafter sonicated at acoustic or ultrasonic frequencies at room or elevated temperature. In the present invention, it has been found that sonication without agitation is convenient. Also, an apparatus for making liposomes, a high pressure homogenizer such as the Microfluidizer®, which can be purchased from Microfluidics Corp., Newton, MA 02164 USA, can be used advantageously. Large volumes of liposome solutions can be prepared with this apparatus under pressures which can reach 600-1200 bar.

In another method, according to the teaching of GB-A-2,134,869 (Squibb), microparticles (10 µm or less) of a hydrosoluble carrier solid (NaCl, sucrose, lactose and other carbohydrates) are coated with an amphipatic agent; the dissolution of the coated carrier in an aqueous phase will yield liposomic vesicles. In GB-A- 2,135,647 insoluble particles, e.g. glass or resin microbeads are coated by moistening in a solution of a lipid in an organic solvent followed by removal of the solvent by evaporation. The lipid coated microbeads are thereafter c ntacted with an aqueous carri r phase, wh reby lip s mic v sicles will f rm in that carrier phase.

The introduction of air or gas into a liposome solution in order to form therein a suspension of microbubbles can be effected by usual means, inter alia by injection, that is, forcing said air or gas through tiny orifices into the liposome solution, or simply dissolving the gas in the solution by applying pressure and thereafter suddenly releasing the pressure. Another way is to agitate or sonicate the liposome solution in the presence of air or an entrappable gas. Also one can generate the formation of a gas within the solution of liposomes itself, for instance by a gas releasing chemical reaction, e.g. decomposing a dissolved carbonate or bicarbonate by acid. The same effect can be obtained by dissolving under pressure a low boiling liquid, for instance butane, in the aqueous phase and thereafter alloving said liquid to boil by suddenly releasing the pressure.

Notwithstanding, an advantageous method is to contact the dry surfactant in lamellar or thin film form with air or an adsorbable or entrappable gas before introducing said surfactant into the liquid carrier phase. In this regard, the method can be derived from the technique disclosed in GB-A-2,135,647, i.e. solid microparticles or beads are dipped in a solution of a film forming surfactant (or mixture of surfactants) in a volatile solvent, after which the solvent is evaporated and the beads are left in contact with air (or an adsorbable gas) for a time sufficient for that air to become superficially bound to the surfactant layer. Thereafter, the beads coated with air filled surfactant are put into a carrier liquid, usually water with or without additives, whereby air bubbles will develop within the liquid by gentle mixing, violent agitation being entirely unnecessary. Then the solid beads can be separated, for instance by filtration, from the microbubble suspension which is remarkably stable with time.

Needless to say that, instead of insoluble beads or spheres, one may use as supporting particles water soluble materials like that disclosed in GB-A- 2,134,869 (carbohydrates or hydrophilic polymers), whereby said supporting particles will eventually dissolve and final separation of a solid becomes un-

necessary. Furthermore in this case, the material of the particles can be selected to eventually act as stabilizer or viscosity enhancer wherever desired.

In a variant of the method, one may also start with dehydrated liposomes, i.e. liposomes which have been prepared normally by means of conventional techniques in the form of aqueous solutions and thereafter dehydrated by usual means, e.g. such as disclosed in US-A- 4,229,360 also incorporated herein as reference. One of the methods for dehydrating liposomes recommended in this reference is freeze-drying (lyophilization), i.e. the liposome solution is frozen and dried by evaporation (sublimation) under reduced pressure. Prior to effecting freeze-drying, a hydrophilic stabilizer compound is dissolved in the solution, for instance a carbohydrate like lactose or sucrose or a hydrophilic polymer like dextran, starch, PVP, PVA and the like. This is useful in the present invention since such hydrophilic compounds also aid in homogenizing the microbubbles size distribution and enhance stability under storage. Actually making very dilute aqueous solutions (0.1 - 10% by weight) of freeze-dried liposomes stabilized with, for instance, a 5:1 to 10:1 weight ratio of lactose to lipid enables to produce aqueous microbubbles suspensions counting 10^8 - 10^9 microbubbles/ml (size distribution mainly 0.5 - 10 µm) which are stable for at least a month (and probably much longer) without significant observable change. And this is obtained by simple dissolution of the air-stored dried liposomes without shaking or any violent agitation. Furthermore, the freeze-drying technique under reduced pressure is very useful because it permits, after drying, to restore the pressure above the dried liposomes with any entrappable gas, i.e. nitrogen, CO_2, argon, methane, freon, etc., whereby after dissolution of the liposomes processed under such conditions suspensions of microbubbles containing the above gases are obtained.

Microbubbles suspensions formed by applying gas pressure on a dilute solution of laminated lipids in water (0.1 - 10% by weight) and thereafter suddenly releasing the pressure have an even higher bubble concentration, e.g. in the order of 10^{10} -

10^{11} bubbles/ml. However, the average bubble size is somewhat above 10 µm, e.g. in the 10 - 50 µm range. In this case, bubble size distribution can be narrowed by centrifugation and layer decantation.

The tensides or surfactants which are convenient in this invention can be selected from all amphipatic compounds capable of forming stable films in the presence of water and gases. The preferred surfactants which can be laminarized include the lecithins (phosphatidyl-choline) and other phospholipids, inter alia phosphatidic acid (PA), phosphatidyl-inositol phosphatidyl-ethanolamine (PE), phosphatidyl-serine (PS), phosphatidyl-glycerol (PG), cardiolipin (CL), sphingomyelins, the plasmogens, the cerebrosides, etc. Examples of suitable lipids are the phospholipids in general, for example, natural lecithins, such as egg lecithin or soya bean lecithin, or synthetic lecithins such as saturated synthetic lecithins, for example, dimyristoyl phosphatidyl choline, dipalmitoyl phosphatidyl choline or distearoyl phosphatidyl choline or unsaturated synthetic lecithins, such as dioleyl phosphatidyl choline or dilinoleyl phosphatidyl choline, with egg lecithin or soya bean lecithin being preferred. Additives like cholesterol and other substances (see below) can be added to one or more of the foregoing lipids in proportions ranging from zero to 50% by weight.

Such additives may include other surfactants that can be used in admixture with the film forming surfactants and most of which are recited in the prior art discussed in the introduction of this specification. For instance, one may cite free fatty acids, esters of fatty acids with polyoxyalkylene compounds like polyoxypropylene glycol and polyoxyethylene glycol; ethers of fatty alcohols with polyoxyalkylene glycols; esters of fatty acids with polyoxyalkylated sorbitan; soaps; glycerol-polyalkylene stearate; glycerol-polyoxyethylene ricinoleate; homo- and copolymers of polyalkylene glycols; polyethoxylated soya-oil and castor oil as well as hydrogenated derivatives; ethers and esters of sucrose or other carbohydrates with fatty acids, fatty alcohols, these being optionally polyoxyalkylated; mono-, di- and triglycerides of saturated or unsaturated fatty acids; glyc-

rides f s ya-oil and sucros . Th am unt f the n n-film forming t nsid s r surfactants can be up t 50% by w ight of the total amount of surfactants in the composition but is preferably between zero and 30%.

The total amount of surfactants relative to the aqueous carrier liquid is best in the range of 0.01 to 25% by weight but quantities in the range 0.5 - 5% are advantageous because one always tries to keep the amount of active substances in an injectable solution as lov as possible, this being to minimize the introduction of foreign materials into living beings even when they are harmless and physiologically compatible.

Further optional additives to the surfactants include:

a) substances which are knovn to provide a negative charg on liposomes, for example, phosphatidic acid, phosphatidyl-glycerol or dicetyl phosphate;

b) substances known to provide a positive charge, for example, stearyl amine, or stearyl amine acetate;

c) substances known to affect the physical properties of the lipid films in a more desirable way; for example, capro-lactam and/or sterols such as cholesterol, ergosterol, phytosterol, sitosterol, sitosterol pyroglutamate, 7-dehydro-cholesterol or lanosterol, may affect lipid films rigidity;

d) substances known to have antioxidant properties to improve the chemical stability of the components in the suspensions, such as tocopherol, propyl gallate, ascorbyl palmitate, or butylated hydroxy-toluene.

The aqueous carrier in this invention is mostly water with possibly small quantities of physiologically compatible liquids such as isopropanol, glycerol, hexanol and the like (see for instance EP-A- 52.575). In general the amount of the organic hydrosoluble liquids will not exceed 5 - 10% by weight.

The present composition may also contain dissolved or suspended therein hydrophilic compounds and polymers defined generally under the name of viscosity enhancers or stabilizers. Although the presence of such compounds is not compulsory for ensuring stability to th air r gas bubbles with tim in th pres nt disp rsi ns, they are advantage us t give s me kind f

"body" to the solutions. When desired, the upper concentrations of such additives when totally innocuous can be very high, for instance up to 80 - 90% by weight of solution with Iopamidol and other iodinated X-ray contrast agents. However with other viscosity enhancers like for instance sugars, e.g. lactose, sucrose, maltose, galactose, glucose, etc. or hydrophilic polymers like starch, dextran, polyvinyl alcohol, polyvinyl-pyrrolidone, dextrin, xanthan or partly hydrolyzed cellulose oligomers, as well as proteins and polypeptides, the concentrations are best between about 1 and 40% by weight, a range of about 5 - 20% being preferred.

Like in the prior art, the injectable compositions of this invention can also contain physiologically acceptable electrolytes; an example is an isotonic solution of salt.

The present invention naturally also includes dry storable pulverulent blends which can generate the present microbubble containing dispersions upon simple admixing with water or an aqueous carrier phase. Preferably such dry blends or formulations will contain all solid ingredients necessary to provide the desired microbubbles suspensions upon the simple addition of water, i.e. principally the surfactants in lamellar form containing trapped or adsorbed therein the air or gas required for microbubble formation, and accessorily the other non-film forming surfactants, the viscosity enhancers and stabilizers and possibly other optional additives. As said before, the air or gas entrappment by the laminated surfactants occurs by simply exposing said surfactants to the air (or gas) at room or superatmospheric pressure for a time sufficient to cause said air or gas to become entrapped within the surfactant. This period of time can be very short, e.g. in the order of a few seconds to a few minutes although over-exposure, i.e. storage under air or under a gaseous atmosphere is in no way harmful. What is important is that air can well contact as much as possible of the available surface of the laminated surfactant, i.e. the dry material should preferably be in a "fluffy" light flowing condition. This is precisely this condition which results from the fore-drying of an aqueous solution of lipos m s and hydr -

philic agent as disclosed in US-A- 4,229,360.

In general, the weight ratio of surfactants to hydrophilic viscosity enhancer in the dry formulations will be in the order of 0.1:10 to 10:1, the further optional ingredients, if any, being present in a ratio not exceeding 50% relative to the total of surfactants plus viscosity enhancers.

The dry blend formulations of this invention can be prepared by very simple methods. As seen before, one preferred method is to first prepare an aqueous solution in which the film forming lipids are laminarized, for instance by sonication, or using any conventional technique commonly used in the liposome field, this solution also containing the other desired additives, i.e. viscosity enhancers, non-film forming surfactants, electrolyte, etc., and thereafter freeze drying to a free flowable powder which is then stored in the presence of air or an entrappable gas.

The dry blend can be kept for any period of time in the dry state and sold as such. For putting it into use, i.e. for preparing a gas or air microbubble suspension for ultrasonic imaging, one simply dissolves a known weight of the dry pulverulent formulation in a sterile aqueous phase, e.g. water or a physiologically acceptable medium. The amount of powder will depend on the desired concentration of bubbles in the injectable product, a count of about 10^8 - 10^9 bubbles/ml being generally that from making a 5 - 20% by weight solution of the powder in water. But naturally this figure is only indicative, the amount of bubbles being essentially dependent on the amount of air or gas trapped during manufacture of the dry powder. The manufacturing steps being under control, the dissolution of the dry formulations will provide microbubble suspensions with well reproducible counts.

The resulting microbubble suspensions (bubble in the 0.5 - 10 µm range) are extraordinarily stable with time, the count originally measured at start staying unchanged or only little changed for weeks and even months; the only observable change is a kind of segregation, the larger bubbles (around 10 µm) tending to rise faster than the smaller ones.

It has also been found that the microbubbles suspensions of this invention can be diluted with very little loss in the number of microbubbles to be expected from dilution, i.e. even in the case of high dilution ratios, e.g. $1/10^2$ to $1/10^4$, the microbubble count reduction accurately matches with the dilution ratio. This indicates that the stability of the bubbles depends on the surfactant in lamellar form rather than on the presence of stabilizers or viscosity enhancers like in the prior art. This property is advantageous in regard to imaging test reproducibility as the bubbles are not affected by dilution with blood upon injection into a patient.

Another advantage of the bubbles of this invention versus the microcapsules of the prior art surrounded by a rigid but breakable membrane which may irreversibly fracture under stress is that when the present suspensions are subject to sudden pressure changes, the present bubbles will momentarily contract elastically and then resume their original shape when the pressure is released. This is important in clinical practice when the microbubbles are pumped through the heart and therefore are exposed to alternating pressure pulses.

The reasons why the microbubbles in this invention are so stable are not clearly understood. Since to prevent bubble escape the buoyancy forces should equilibrate with the retaining forces due to friction, i.e. to viscosity, it is theorized that the bubbles are probably surrounded by the laminated surfactant. Whether this laminar surfactant is in the form of a continuous or discontinuous membrane, or even as closed spheres attached to the microbubbles, is for the moment unknown but under investigation. However the lack of a detailed knowledge of the phenomena presently involved does not preclude full industrial operability of the present invention.

The bubble suspensions of the present invention are also useful in other medical/diagnostic applications where it is desirable to target the stabilised microbubbles to specific sites in the body following their injection, for instance to thrombi present in blood vessels, atherosclerotic lesions (plaques) in arteries, tumor cells, as well as for th

diagnosis of altered surfaces of body cavities, e.g. ulceration sites in the stomach or tumors of the bladder. For this, one can bind monoclonal antibodies tailored by genetic engineering, antibody fragments or polypeptides designed to mimic antibodies, bioadhesive polymers, lectins and other site-recognizing molecules to the surfactant layer stabilizing the microbubbles. Thus monoclonal antibodies can be bound to phospholipid bilayers by the method described by L.D. Leserman, P. Machy and J. Barbet ("Liposome Technology vol. III" p. 29 ed. by G. Gregoriadis, CRC Press 1984). In another approach a palmitoyl antibody is first synthesized and then incorporated in phospholipid bilayers following L. Huang, A. Huang and S.J. Kennel ("Liposome Technology vol. III" p. 51 ed. by G. Gregoriadis, CRC Press 1984). Alternatively, some of the phospholipids used in the present invention can be carefully selected in order to obtain preferential uptake in organs or tissues or increased half-life in blood. Thus GM1 gangliosides- or phosphatidylinositol-containing liposomes, preferably in addition to cholesterol, will lead to increased half-lifes in blood after intravenous administration in analogy with A. Gabizon, D. Papahadjopoulos, Proc. Natl Acad. Sci USA 85 (1988) 6949.

The gases in the microbubbles of the present invention can include, in addition to current innocuous physiologically acceptable gases like CO_2, nitrogen, N_2O, methane, butane, freon and mixtures thereof, radioactive gases such as ^{133}Xe or ^{81}Kr are of particular interest in nuclear medicine for blood circulation measurements, for lung scintigraphy etc.

The following Examples illustrate the invention on a practical stand-point.

Echogenic measurements.

Echogenicity measurements were performed in a pulse - echo system made of a plexiglas specimen holder (diameter 30 mm) and a transducer holder immersed in a constant temperature water bath, a pulser-receiver (Accutron M3010S) with for the receiving part an external pre-amplifier with a fixed gain of 40 dB and an internal amplifier with adjustabl gain from -40 t +40 dB. A

10MHz low-pass filter was inserted in the receiving part to improve the signal to noise ratio. The A/D board in the IBM PC was a Sonotek STR 832. Measurements were carried out at 2.25, 3.5, 5 and 7.5 MHz.

Example 1

A liposome solution (50 mg lipids per ml) was prepared in distilled water by the REV method (see F. Szoka Jr. and D. Papahadjopoulos, Proc. Natl. Acad. Sci. USA 75 (1978) 4194) using hydrogenated soya lecithin (HC 95 H, Nattermann Chemie, Köln, W. Germany) and dicetylphosphate in a molar ratio 9/1. This liposome preparation was extruded at 65°C (to calibrate the vesicle size) through a 1 µm polycarbonate filter (Nucleopore). Two ml of this solution were admixed with 5 ml of a 75%-iopamidol solution in water and 0.4 ml of air and the mixture was forced back and forth through a two syringe system as disclosed in DE-A-3529195, while maintaining continuously a slight over-pressure. This resulted in the formation of a suspension of microbubbles of air in the liquid (10^5-10^6 bubbles per ml, bubble size 1-20 µm as estimated by light microscopy) which was stable for several hours at room temperature. This suspension gave a strong echo signal when tested by ultrasonic echography at 7.5, 5, 3.5 and 2.25 MHz.

Example 2

A distilled water solution (100 ml) containing by weight 2% of hydrogenated soya lecithin and dicetylphosphate in a 9/1 molar ratio was sonicated for 15 min at 60-65°C with a Branson probe sonifier (Type 250).

After cooling, the solution was centrifuged for 15 min at 10,000 g and the supernatant was recovered and lactose added to make a 7.5% b.v. solution. The solution was placed in a tight container in which a pressure of 4 bar of nitrogen was established for a few minutes while shaking the container. Afterwards, the pressure was released suddenly whereby a highly concentrated bubble suspension was obtained (10^{10} - 10^{11} bubbles/ml). The size distribution of the bubbles was however

wid r than in Example 1, i.e. fr m ab ut 1 t 50 µm. The suspension was v ry stabl but after a few days a segregation occurred in the standing phase, the larger bubbles tending to concentrate in the upper layers of the suspension.

Example 3

Twenty g of glass beads (diameter about 1 mm) were immersed into a solution of 100 mg of dipalmitoylphosphatidylcholin (Fluka A.G. Buchs) in 10 ml of chloroform. The beads were rotated under reduced pressure in a rotating evaporator until all $CHCl_3$ had escaped. Then the beads were further rotated under atmospheric pressure for a few minutes and 10 ml of distilled water were added. The beads were removed and a suspension of air microbubbles was obtained which was shown to contain about 10^6 bubbles/ml after examination under the microscope. The average size of the bubbles was about 3 - 5 µm. The suspension was stable for several days at least.

Example 4

A hydrogenated soya lecithin/dicetylphosphate suspension in water was laminarized using the REV technique as described in Example 1. Two ml of the liposome preparation were added to 8 ml of 15% maltose solution in distilled water. The resulting solution was frozen at -30°C, then lyophilized under 0.1 Torr. Complete sublimation of the ice was obtained in a few hours. Thereafter, air pressure was restored in the evacuated container so that the lyophilized powder became saturated with air in a few minutes.

The dry powder was then dissolved in 10 ml of sterile water under gentle mixing, whereby a microbubble suspension (10^8-10^9 microbubbles per ml, dynamic viscosity < 20 mPa.s) was obtained. This suspension containing mostly bubbles in the 1-5 µm range was stable for a very long period, as numerous bubbles could still be detected after 2 months standing. This microbubble suspension gave a strong response in ultrasonic echography. If in this example th s luti n is froz n by spraying in air at -30 t -70°C t btain a frozen sn w instead f a m n lithic bl ck

and the sn v is then evaporated under vacuum, excellent results are obtained.

Example 5

Two ml samples of the liposome solution obtained as described in Example 4 were mixed with 10 ml of an 5% aqueous solution of gelatin (sample 5A), human albumin (sample 5B), dextran (sample 5C) and iopamidol (sample 5D). All samples were lyophilized. After lyophilization and introduction of air, the various samples were gently mixed with 20 ml of sterile water. In all cases, the bubble concentration was above 10^8 bubbles per ml and almost all bubbles were below 10 µm. The procedure of the foregoing Example was repeated with 9 ml of the liposome preparation (450 mg of lipids) and only one ml of a 5% human albumin solution. After lyophilization, exposure to air and addition of sterile water (20 ml), the resulting solution contained 2 x 10^8 bubbles per ml, most of them below 10 µm.

Example 6

Lactose (500 mg), finely milled to a particle size of 1-3 µm, was moistened with a chloroform (5 ml) solution of 100 mg of dimyristoylphosphatidylcholine/cholesterol/dipalmitoylphosphatidi acid (from Fluka) in a molar ratio of 4:1:1 and thereafter evaporated under vacuum in a rotating evaporator. The resulting free floving white povder vas rotated a fev minutes under nitrogen at normal pressure and thereafter dissolved in 20 ml of sterile vater. A microbubble suspension vas obtained vith about 10^5-10^6 microbubbles per ml in the 1-10 µm size range as ascertained by observation under the microscope. In this Example, the veight ratio of coated surfactant to vater-soluble carrier vas 1:5. Excellent results (10^7-10^8 microbubbles/ml) are also obtained vhen reducing this ratio to lover values, i.e. down to 1:20, vhich vill actually increase the surfactant efficiency for the intake of air, that is, this vill decrease th veight f surfactant nec ssary f r pr ducing th sam bubbl c unt.

Example 7.

An aqueous solution containing 2% of hydrogenated soya lecithin and 0.4% of Pluronic® F68 (a non ionic polyoxyethylene-polyoxypropylene copolymer surfactant) was sonicated as described in Example 2. After cooling and centrifugation, 5 ml of this solution were added to 5 ml of a 15% maltose solution in water. The resulting solution was frozen at -30°C and evaporated under 0.1 Torr. Then air pressure was restored in the vessel containing the dry powder. This was left to stand in air for a few seconds, after which it was used to make a 10% by weight aqueous solution which showed under the microscope to be a suspension of very tiny bubbles (below 10 μm); the bubble concentration was in the range of 10^7 bubbles per ml. This preparation gave a very strong response in ultrasonic echography at 2.25, 3.5, 5 and 7.5 MHz.

Example 8

Two-dimensional echocardiography was performed in an experimental dog following peripheral vein injection of 0.1-2 ml of the preparation obtained in Example 4. Opacification of the left heart with clear outlining of the endocardium was observed, thereby confirming that the microbubbles (or at least a significant part of them) were able to cross the pulmonary capillary circulation.

Example 9

A phospholipid/maltose lyophilized powder was prepared as described in Example 4. However, at the end of the lyophilization step, a ^{133}Xe containing gas mixture was introduced in the evacuated container instead of air. A few minutes later, sterile water was introduced and after gentle mixing a microbubble suspension containing ^{133}Xe in the gas phase was produced. This microbubble suspension was injected into living bodies to undertake investigations requiring use of ^{133}Xe as tracer. Excellent results were obtained.

Example 10 (comparative)

In US-A-4,900,540, Ryan et al disclose gas filled liposomes for ultrasonic investigations. According to the citation, liposomes are formed by conventional means but with the addition of a gas or gas precursor in the aqueous composition forming the liposome core (col. 2, lines 15-27).

Using a gas precursor (bicarbonate) is detailed in Examples 1 and 2 of the reference. Using an aqueous carrier with an added gas for encapsulating the gas in the liposomes (not examplifi d by Ryan et al) will require that the gas be in the form of very small bubbles, i.e. of size similar or smaller than the size f the liposome vesicles.

Aqueous media in which air can be entrapped in the form of very small bubbles (2.5-5 µm) are disclosed in M.W. Keller et al, J. Ultrasound Med. 5 (1986), 413-498.

A quantity of 126 mg of egg lecithin and 27 mg of cholesterol were dissolved in 9 ml of chloroform in a 200 ml round bottom flask. The solution of lipids was evaporated to dryness on a Rotavapor whereby a film of the lipids was formed on the walls of the flask. A 10 ml of a 50% by weight aqueous dextrose solution was sonicated for 5 min according to M.W. Keller et al (ibid) to generate air microbubbles therein and th sonicated solution was added to the flask containing the film of lipid, whereby hand agitation of the vessel resulted int hydration of the phospholipids and formation of multilamellar liposomes within the bubbles containing carrier liquid.

After standing for a while, the resulting liposome suspension was subjected to centrifugation under 5000 g for 15 min to remove from the carrier the air not entrapped in the vesicles. It was also expected that during centrifugation, the air filled liposomes would segregate to the surface by buoyancy.

After centrifugation the tubes were examined and showed a bottom residue consisting of agglomerated dextrose filled liposomes and a clear supernatant liquid with substantially no bubble l ft. The quantity f air filled lip s mes having risen by buoyancy was n gligibly small and c uld n t b ascertained.

Example 11 (Comparative)

An injectable contrast composition was prepared according to Ryan (US-A-4,900,540, col. 3, Example 1). Egg lecithin (126 mg) and cholesterol (27 mg) were dissolved in 9 ml of diethylether. To the solution were added 3 ml of 0.2 molar aqueous bicarbonate and the resulting two phase systems was sonicated until becoming homogeneous. The mixture was evaporated in a Rotavapor apparatus and 3 ml of 0.2 molar aqueous bicarbonate were added.

A 1 ml portion of the liposome suspension was injected int the jugular vein of an experimental rabbit, the animal being under condition for heart ultrasonic imaging using an Acuson 128-XP5 ultrasonic imager (7.5 transducer probe for imaging th heart). The probe provided a cross-sectional image of the right and left ventricles (mid-papillary muscle). After injection, a light and transient (a few seconds) increase in the outline of the right ventricle was observed. The effect was however much inferior to the effect observed using the preparation of Example 4. No improvement of the imaging of the left ventricle was noted which probably indicates that the CO_2 loaded liposomes did not pass the pulmonary capillaries barrier.

CLAIMS

1. A composition adapted for injection into the blood-stream and body cavities of living beings, e.g. for the purpose of ultrasonic echography, consisting of a suspension of air or gas microbubbles in a physiologically acceptable aqueous carrier phase comprising from about 0.01 to about 20% by weight of one or more dissolved or dispersed surfactants, characterized in that at least one of the surfactants is a film forming surfactant present in the composition at least partially in lamellar or laminar form.

2. The composition of claim 1, characterized in that the lamellar surfactant is in the form of mono- or pluri-molecular membrane layers.

3. The composition of claim 1, characterized in that the lamellar surfactant is in the form of liposome vesicles.

4. The composition of claim 1, characterized in that it essentially consists of a liposome solution containing air or gas microbubbles developed therein.

5. The composition of claim 4, characterized in that the size of most of both liposomes and microbubbles is below 50 µm, preferably below 10 µm.

6. The composition of claim 1, containing about 10^8 - 10^9 bubbles of 0.5 - 10 µm size/ml, said concentration showing little or substantially no variability under storage for at least a month.

7. The composition of claim 1, characterized in that the surfactants are selected from phospholipids including the lecithins such as phosphatidic acid, phosphatidyl-choline, phosphatidyl-ethanolamine, phosphatidyl-serine, phosphatidyl-glycerol phosphatidyl-inositol, cardiolipin and sphyngomyelin.

8. The composition of claim 7, characterized in further containing substances affecting the properties of liposomes selected from phosphatidyl-glycerol, dicetyl-phosphate, cholesterol, ergosterol, phytosterol, sitosterol, lanosterol, tocopherol, propyl gallate, ascorbyl palmitate and butylated hydroxytoluene.

9. The composition of claim 1, further containing dissolved viscosity enhancers or stabilizers selected from linear and cross-linked poly- and oligo-saccharides, sugars, hydrophilic polymers and iodinated compounds such as Iopamidol in a weight ratio to the surfactants comprised between about 1:5 to 100:1.

10. The composition of claim 1, in which the surfactants comprise up to 50% by weight of non-laminar surfactants selected from fatty acids, esters and ethers of fatty acids and alcohols with polyols such as polyalkylene glycols, polyalkylenated sugars and other carbohydrates, and polyalkylenated glycerol.

11. A method for the preparation of the suspensions of claim 1, characterized by the following steps:

(a) selecting at least one film forming surfactant and converting it into lamellar form;

(b) contacting the surfactant in lamellar form with air or an adsorbable or entrappable gas for a time sufficient for that air or gas to become bound by said surfactant; and

(c) admixing the surfactant in lamellar form with an aqueous liquid carrier, whereby a stable dispersion of air or gas microbubbles in said liquid carrier will result.

12. The method of claim 11, in which step (c) is brought about before step (b), the latter being effected by introducing pressurized air or gas into the liquid carrier and thereafter releasing the pressure.

13. The method of claim 11, in which step (c) is brought about by gentle mixing of the components, no shaking being necessary, whereby the air or gas bound to the lamellar surfactant in step (b) will develop into a suspension of stable microbubbles.

14. The method of claims 11 or 12, in which the liquid carrier contains dissolved therein stabilizer compounds selected from hydrosoluble proteins, polypeptides, sugars, poly- and oligo-saccharides and hydrophilic polymers.

15. The method of claim 11, in which the conversion of step (a) is effected by coating the surfactant onto particles of a lubricating insoluble materials; step (b) is effected by letting the coated particles stand for a while under air or a gas; and

step (c) is effected by admixing the c ated particles with an aqueous liquid carrier.

16. The method of claim 11, in which the conversion of step (a) is effected by sonicating or homogenizing under high pressure an aqueous solution of film forming lipids, this operation leading, at least partly, to the formation of liposomes.

17. The method of claim 16, in which step (b) is effected by freeze-drying the liposome containing solution, the latter optionally containing hydrophilic stabilizers and contacting the resulting freeze-dried product with air or a gas for a period of time.

18. The method of claims 16 and 17, in which the vater solution of film forming lipids also contains viscosity enhancers or stabilizers selected from hydrophilic polymers and carbohydrates in weight ratio relative to the lipids comprised between 1:5 and 100:1.

19. A dry pulverulent formulation which, upon dissolution in vater, vill form an aqueous suspension of microbubbles for ultrasonic echography, characterized in containing one or more film forming surfactants in laminar form and hydrosoluble stabilizers.

20. The dry formulation of claim 19, in which the surfactants in laminar form are in the form of fine layers deposited on the surface of soluble or insoluble solid particulate material.

21. The dry formulation of claim 20, in which the insoluble solid particles are glass or polymer beads.

22. The dry formulation of claim 20, in which the soluble particles are made of hydrosoluble carbohydrates, polysaccharides, synthetic polymers, albumin, gelatin or Iopamidol.

23. The dry formulation of claim 19, which comprises freeze-dried liposomes.

24. The use of the injectable composition of claim 1 for ultras nic ech graphy.

25. The use f the inje table c mp siti n f claims 1-10 f r transp rting in the bl d-str am or b dy caviti s bubbl s f

foreign gases active therapeutically or diagnostically.

26. The composition of claim 4, in which the surfactant comprises, bound thereto, bioactive species designed for specific targeting purposes, e.g. for immobilising the bubbles in specifically defined sites in the circulatory system, or in organs, or in tissues.

27. The composition of claim 4, in which the surfactant comprises, bound thereto, bioactive species selected from monoclonal antibodies, antibody fragments or polypeptides designed to mimic antibodies, bioadhesive polymers, lectins and other receptor recognizing molecules.

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(54) Polymeric gas or air filled microballoons usable as suspensions in liquid carriers for ultrasonic echography.

(57) Air or gas filled microballoons bounded by an interfacially deposited polymer membrane which can be dispersed in aqueous carrier liquids to be injected into living organisms or administered orally, rectally and urethrally for therapeutic or diagnostic purposes (echography). The properties of the polymeric membrane of the microballoons (elasticity, permeability, biodegradability) can be controlled at will depending on the selected polymer, the interfacial deposition conditions, and the polymer additives.

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The present invention concerns air or gas filled microcapsules or microballoons enclosed by an organic polymer envelope which can be dispersed or suspended in aqueous media and used in this form for oral, rectal and urethral applications or for injection into living beings, for instance for the purpose of ultrasonic echography and other medical applications.

The invention also comprises a method for making said microballoons in the dry state, the latter being instantly dispersible in an aqueous liquid carrier to give suspensions with improved properties over existing similar products. Hence, suspensions of the microballoons in a carrier liquid ready for administration are also part of the invention.

It is well known that microbodies or microglobules of air or a gas, e.g. microspheres like microbubbles or microballoons, suspended in a liquid are exceptionally efficient ultrasound reflectors for echography. In this disclosure the term of "microbubble" specifically designates air or gas microspheres in suspension in a carrier liquid which generally result from the introduction therein of air or a gas in divided form, the liquid preferably also containing surfactants or tensides to control the surface properties and the stability of the bubbles. In the microbubbles, the gas to liquid interface essentially comprises loosely bound molecules of the carrier liquid. The term of "microcapsule" or "microballoon" designates preferably air or gas bodies with a material boundary or envelope of molecules other than that of the carrier liquid, i.e. a polymer membrane wall. Both microbubbles and microballoons are useful as ultrasonic contrast agents. For instance injecting into the bloodstream of living bodies suspensions of gas microbubbles or microballoons (in the range of 0.5 to 10 µm) in a carrier liquid will strongly reinforce ultrasonic echography imaging, thus aiding in the visualization of internal organs. Imaging of vessels and internal organs can strongly help in medical diagnosis, for instance for the detection of cardiovascular and other diseases.

The formation of suspensions of microbubbles in an injectable liquid carrier suitable for echography can be produced by the release of a gas dissolved under pressure in this liquid, or by a chemical reaction generating gaseous products, or by admixing with the liquid soluble or insoluble solids containing air or gas trapped or adsorbed therein.

For instance, in US-A-4,446,442 (Schering), there are disclosed a series of different techniques for producing suspensions of gas microbubbles in a sterilized injectable liquid carrier using (a) a solution of a tenside (surfactant) in a carrier liquid (aqueous) and (b) a solution of a viscosity enhancer as stabilizer. For generating the bubbles, the techniques disclosed there include forcing at high velocity a mixture of (a), (b) and air through a small aperture; or injecting (a) into (b) shortly before use together with a physiologically acceptable gas; or adding an acid to (a) and a carbonate to (b), both components being mixed together just before use and the acid reacting with the carbonate to generate CO_2 bubbles; or adding an over-pressurized gas to a mixture of (a) and (b) under storage, said gas being released into microbubbles at the time when the mixture is used for injection.

One problem with microbubbles is that they are generally short-lived even in the presence of stabilizers. Thus, in EP-A-131.540 (Schering), there is disclosed the preparation of microbubble suspensions in which a stabilized injectable carrier liquid, e.g. a physiological aqueous solution of salt, or a solution of a sugar like maltose, dextrose, lactose or galactose, is mixed with solid microparticles (in the 0.1 to 1 µm range) of the same sugars containing entrapped air. In order to develop the suspension of bubbles in the liquid carrier, both liquid and solid components are agitated together under sterile conditions for a few seconds and, once made, the suspension must then be used immediately, i.e. it should be injected within 5-10 minutes for echographic measurements; indeed, because the bubbles are evanescent, the concentration thereof becomes too low for being practical after that period.

Another problem with microbubbles for echography after injection is size. As commonly admitted, microbubbles of useful size for allowing easy transfer through small blood vessels range from about 0.5 to 10 µm; with larger bubbles, there are risks of clots and consecutive emboly. For instance, in the bubble suspensions disclosed in US-A-4,446,442 (Schering) in which aqueous solutions of surfactants such as lecithin, esters and ethers of fatty acids and fatty alcohols with polyoxyethylene and polyoxyethylated polyols like sorbitol, glycols and glycerol, cholesterol, or polyoxy-ethylenepolyoxypropylene polymers, are vigorously shaken with solutions of viscosity raising and stabilizing compounds such as mono- and polysaccharides (glucose, lactose, sucrose, dextran, sorbitol); polyols, e.g. glycerol, polyglycols; and polypeptides like proteins, gelatin, oxypolygelatin and plasma protein, only about 50% of the microbubbles are below 40-60 µm which makes such suspensions unsuitable in many echographic application.

In contrast, microcapsules or microballoons have been developed in an attempt to cure some or the foregoing deficiencies. As said before, while the microbubbles only have an immaterial or evanescent envelope, i.e. they are only surrounded by a wall of liquid whose surface tension is being modified by the presence of a surfactant, the microballoons or microcapsules have a tangible envelope made of substantive material other than the carrier itself, e.g. a polymeric membrane with definite mechanical strength. In other terms, they are microspheres of solid material in which the air or gas is more or less

tightly encapsulated.

For instance, US-A-4,276,885 (Tickner et al.) discloses using surface membrane microcapsules containing a gas for enhancing ultrasonic images, the membrane including a multiplicity of non-toxic and non-antigenic organic molecules. In a disclosed embodiment, these microbubbles have a gelatin membrane which resists coalescence and their preferred size is 5-10 µm. The membrane of these microbubbles is said to be sufficiently stable for making echographic measurements; however it is also said that after a period of time the gas entrapped therein will dissolve in the blood-stream and the bubbles will gradually disappear, this being probably due to slow dissolution of the gelatin. Before use, the microcapsules are kept in gelatin solutions in which they are storage stable, but the gelatin needs to be heated and melted to become liquid at the time the suspension is used for making injection.

Microspheres of improved storage stability although without gelatin are disclosed in US-A-4,718,433 (Feinstein). These microspheres are made by sonication (5 to 30 RHz) of viscous protein solutions like 5% serum albumin and have diameters in the 2-20 µm range, mainly 2-4 µm. The microspheres are stabilized by denaturation of the membrane forming protein after sonication, for instance by using heat or by chemical means, e.g. by reaction with formaldehyde or glutaraldehyde. The concentration of stable microspheres obtained by this technique is said to be about 8×10^8/ml in the 2-4 µm range, about 10^8/ml in the 4-6 µm range and less than 5×10^5 in the 6-8 µm range. The stability time of these microspheres is said to be 48 hrs or longer and they permit convenient left heart imaging after intravenous injection. For instance, the sonicated albumin microbubbles when injected into a peripheral vein are capable of transpulmonary passage. This results in echocardiographic opacification of the left ventricle cavity as well as myocardial tissues.

Recently still further improved microballoons for injection ultrasonic echography have been reported in EP-A-324,938 (Widder). In this document there are disclosed high concentrations (more than 10^9) of air-filled protein-bounded microspheres of less than 10 µm which have life-times of several months or more. Aqueous suspensions of these microballoons are produced by ultrasonic cavitation of solutions of denaturable proteins, e.g. human serum albumin, which operation also leads to a degree of foaming of the membrane-forming protein and its subsequent hardening by heat. Other proteins such as hemoglobin and collagen are said to be convenient also.

Still more recently M.A. Wheatley et al., Biomaterials 11 (1990), 713-717, have reported the preparation of polymer-coated microspheres by ionotropic gelation of alginate. The reference mentions several techniques to generate the microcapsules; in one case an alginate solution was forced through a needle in an air jet which produced a spray of nascent air filled capsules which were hardened in a bath of 1.2% aqueous $CaCl_2$. In a second case involving co-extrusion of gas and liquid, gas bubbles were introduced into nascent capsules by means of a triple-barelled head, i.e. air was injected into a central capillary tube while an alginate solution was forced through a larger tube arranged coaxially with the capillary tube, and sterile air was flown around it through a mantle surrounding the second tube. Also in a third case, gas was trapped in the alginate solution before spraying either by using a homogenizer or by sonication. The microballoons thus obtained had diameters in the range 30-100 µm, however still oversized for easily passing through lung capillaries.

The high storage stability of the suspensions of microballoons disclosed in EP-A-324,938 enables them to be marketed as such, i.e. with the liquid carrier phase, which is a strong commercial asset since preparation before use is no longer necessary. However, the protein material used in this document may cause allergenic reactions with sensitive patients and, moreover, the extreme strength and stability of the membrane material has some drawbacks: for instance, because of their rigidity, the membranes cannot sustain sudden pressure variations to which the microspheres can be subjected, for instance during travel through the blood-stream, these variations of pressure being due to heart pulsations. Thus, under practical ultrasonic tests, a proportion of the microspheres will be ruptured which makes imaging reproducibility awkward; also, these microballoons are not suitable for oral application as they will not resist the digestive enzymes present in the gastrointestinal tract. Moreover, it is known that microspheres with flexible walls are more echogenic than corresponding microspheres with rigid walls.

Furthermore, in the case of injections, excessive stability of the material forming the walls of the microspheres will slow down its biodegradation by the organism under test and may result into metabolization problems. Hence it is much preferable to develop pressure sustaining microballoons bounded by a soft and elastic membrane which can temporarily deform under variations of pressure and endowed with enhanced echogenicity; also it might be visualized that micro-balloons with controllable biodegradability, for instance made of semi-permeable biodegradable polymers with controlled microporosity for allowing slow penetration of biological liquids, would be highly advantageous.

These desirable features have now been achieved with the microballoons of the present invention as defined in claims 1 and 2, and subsequent claims. Moreover, although the present microspheres can generally be made relatively short-lived, i.e. susceptible to biodegradation to cope with the foregoing

metabolization problems by using selected types of polymers, this feature (which is actually controlled by the fabrication parameters) is not a commercial drawback because either the microballoons can be stored and shipped dry, a condition in which they are stable indefinitely, or the membrane can be made substantially impervious to the carrier liquid, degradation starting to occur only after injection. In the first case, the microballoons supplied in dry powder form are simply admixed with a proportion of an aqueous phase carrier before use, this proportion being selected depending on the needs. Note that this is an additional advantage over the prior art products because the concentration can be chosen at will and initial values far exceeding the aforementioned 10^9/ml, i.e. in the range 10^6 to 10^{10}, are readily accessible. It should be noted that the method of the invention (to be disclosed hereafter) enables to control porosity to a wide extent; hence microballoons with a substantially impervious membrane can be made easily which are stable in the form of suspensions in aqueous liquids and which can be marketed as such also.

Microspheres with membranes of interfacially deposited polymers as defined in claim 1, although in the state where they are filled with liquid, are well known in the art. They may normally result from the emulsification into droplets (the size of which is controllable in function to the emulsification parameters) of a first aqueous phase in an organic solution of polymer followed by dispersion of this emulsion into a second water phase and subsequent evaporation of the organic solvent. During evaporation of the volatile solvent, the polymer deposits interfacially at the droplets boundary and forms a microporous membrane which efficiently bounds the encapsulated first aqueous phase from the surrounding second aqueous phase. This technique, although possible, is not preferred in the present invention.

Alternatively, one may emulsify with an emulsifier a hydrophobic phase in an aqueous phase (usually containing viscosity increasing agents as emulsion stabilizers) thus obtaining an oil-in-water type emulsion of droplets of the hydrophobic phase and thereafter adding thereto a membrane forming polymer dissolved in a volatile organic solvent not miscible with the aqueous phase.

If the polymer is insoluble in the hydrophobic phase, it will deposit interfacially at the boundary between the droplets and the aqueous phase. Otherwise, evaporation of the volatile solvent will lead to the formation of said interfacially deposited membrane around the droplets of the emulsified hydrophobic phase. Subsequent evaporation of the encapsulated volatile hydrophobic phase provides water filled microspheres surrounded by interfacially deposited polymer membranes. This technique which is advantageously used in the present invention is disclosed by K. Uno et al. in J. Microencapsulation 1 (1984), 3-8 and K. Makino et al., Chem. Pharm. Bull. 33 (1984), 1195-1201. As said before, the size of the droplets can be controlled by changing the emulsification parameters, i.e. nature of emulsifier (more effective the surfactant, i.e. the larger the hydrophilic to lipophilic balance, the smaller the droplets) and the stirring conditions (faster and more energetic the agitation, the smaller the droplets).

In another variant, the interfacial wall forming polymer is dissolved in the starting hydrophobic phase itself; the latter is emulsified into droplets in the aqueous phase and the membrane around the droplets will form upon subsequent evaporation of this encapsulated hydrophobic phase. An example of this is reported by J.R. Fernand et al., Powder Technology 22 (1979), 11-16 who emulsify a solution of polymer (e.g. polyethylene) in naphthalene in boiling water, then after cooling they recover the naphthalene in the form of a suspension of polymer bounded microbeads in cold water and, finally, they remove the naphthalene by subjecting the microbeads to sublimation, whereby 25 µm microballoons are produced. Other examples exist, in which a polymer is dissolved in a mixed hydrophobic phase comprising a volatile hydrophobic organic solvent and a water-soluble organic solvent, then this polymer solution is emulsified in a water phase containing an emulsifier, whereby the water-soluble solvent disperses into the water phase, thus aiding in the formation of the emulsion of microdroplets of the hydrophobic phase and causing the polymer to precipitate at the interface; this is disclosed in EP-A-274.961 (H. Fessi).

The aforementioned techniques can be adapted to the preparation of air or gas filled microballoons suited for ultrasonic imaging provided that appropriate conditions are found to control sphere size in the desired ranges, cell-wall permeability or imperviousness and replacement of the encapsulated liquid phase by air or a selected gas. Control of overall sphere size is obviously important to adapt the microballoons to use purposes, i.e. injection or oral intake. The size conditions for injection (about 0.8 - 10 µm average size) have been discussed previously. For oral application, the range can be much wider, being considered that echogenicity increases with size; hence microballoons in several size ranges between say 1 and 1000 µm can be used depending on the needs and provided the membrane is elastic enough not to break during transit in the stomach and intestine. Control of cell-wall permeability is important to ensure that infiltration by the injectable aqueous carrier phase is absent or slow enough not to impair the echographic measurements but, in cases, still substantial to ensure relatively fast after-test biodegradability, i.e. ready metabolization of the suspension by the organism. Also the microporous structure of the microballoons envelope (pores of a few nm to a few

hundreds of nm or more for microballoons envelopes of thickness ranging from 50-600 nm) is a factor of resiliency, i.e. the microspheres can readily accept pressure variations without breaking. The preferred range of pore sizes is about 50-2000 nm.

The conditions for achieving these results are met by using the method disclosed in claims 17, 18 and subsequent claims.

One factor which enables to control the permeability of the microballoons membrane is the rate of evaporation of the hydrophobic phase relative to that of water in step (4) of the method of claim 17, e.g. under conditions of freeze drying which is the case of the embodiment recited in claim 20. For instance if the evaporation is carried out between about -40 and 0°C, and hexane is used as the hydrophobic phase, polystyrene being the interfacially deposited polymer, beads with relatively large pores are obtained; this is so because the vapour pressure of the hydrocarbon in the chosen temperature range is significantly greater than that of water, which means that the pressure difference between the inside and outside of the spheres will tend to increase the size of the pores in the spheres membrane through which the inside material will be evaporated. In contrast, using cyclooctane as the hydrophobic phase (at -17°C the vapour pressure is the same as that of water) will provide beads with very tiny pores because the difference of pressures between the inside and outside of the spheres during evaporation is minimized.

Depending on degree of porosity the microballoons of this invention can be made stable in an aqueous carrier from several hours to several months and give reproducible echographic signals for a long period of time. Actually, depending on the polymer selected, the membrane of the microballoons can be made substantially impervious when suspended in carrier liquids of appropriate osmotic properties, i.e. containing solutes in appropriate concentrations. It should be noted that the existence of micropores in the envelope of the microballoons of the present invention appears to be also related with the echographic response, i.e., all other factors being constant, microporous vesicles provide more efficient echographic signal than corresponding non-porous vesicles. The reason is not known but it can be postulated that when a gas is in resonance in a closed structure, the damping properties of the latter may be different if it is porous or non-porous.

Other non water soluble organic solvents which have a vapour pressure of the same order of magnitude between about -40°C and 0°C are convenient as hydrophobic solvents in this invention. These include hydrocarbons such as for instance n-octane, cyclooctane, the dimethylcyclohexanes, ethyl-cyclohexane, 2-, 3- and 4-methyl-heptane, 3-ethyl-hexane, toluene, xylene, 2-methyl-2-heptane, 2,2,3,3-tetramethylbutane and the like. Esters such as propyl and isopropyl butyrate and isobutyrate, butyl-formate and the like, are also convenient in this range. Another advantage of freeze drying is to operate under reduced pressure of a gas instead of air, whereby gas filled microballoons will result. Physiologically acceptable gases such as CO_2, N_2O, methane, Freon, helium and other rare gases are possible. Gases with radioactive tracer activity can be contemplated.

As the volatile solvent insoluble in water to be used for dissolving the polymer to be precipitated interfacially, one can cite halo-compounds such as CCl_4, CH_2Br, CH_2Cl_2, chloroform, Freon, low boiling esters such as methyl, ethyl and propyl acetate as well as lower ethers and ketones of low water solubility. When solvents not totally insoluble in water are used, e.g. diethyl-ether, it is advantageous to use, as the aqueous phase, a water solution saturated with said solvent beforehand.

The aqueous phase in which the hydrophobic phase is emulsified as an oil-in-water emulsion preferably contains 1-20% by weight of water-soluble hydrophilic compounds like sugars and polymers as stabilizers, e.g. polyvinyl alcohol (PVA), polyvinyl pyrrolidone (PVP), polyethylene glycol (PEG), gelatin, polyglutamic acid, albumin, and polysaccharides such as starch, dextran, agar, xanthan and the like. Similar aqueous phases can be used as the carrier liquid in which the microballoons are suspended before use.

Part of this water-soluble polymer can remain in the envelope of the microballoons or it can be removed by washing the beads before subjecting them to final evaporation of the encapsulated hydrophobic core phase.

The emulsifiers to be used (0.1-5% by weight) to provide the oil-in-water emulsion of the hydrophobic phase in the aqueous phase include most physiologically acceptable emulsifiers, for instance egg lecithin or soya bean lecithin, or synthetic lecithins such as saturated synthetic lecithins, for example, dimyristoyl phosphatidyl choline, dipalmitoyl phosphatidyl choline or distearoyl phosphatidyl choline or unsaturated synthetic lecithins, such as dioleyl phosphatidyl choline or dilinoleyl phosphatidyl choline. Emulsifiers also include surfactants such as free fatty acids, esters of fatty acids with polyoxyalkylene compounds like polyoxypropylene glycol and polyoxyethylene glycol; ethers of fatty alcohols with polyoxyalkylene glycols; esters of fatty acids with polyoxyalkylated sorbitan; soaps; glycerol-polyalkylene stearate; glycerol-polyoxyethylene ricinoleate; homo- and copolymers of polyalkylene glycols; polyethoxylated soya-oil and castor oil as well as hydrogenated derivatives; ethers and esters of sucrose or other carbohydrates with fatty acids, fatty alcohols, these being optionally polyoxyalkylated; mono-, di- and triglycerides of saturated or unsaturated fatty acids;

glycerides or soya-oil and sucrose.

The polymer which constitutes the envelope or bounding membrane of the injectable microballoons can be selected from most hydrophilic, biodegradable physiologically compatible polymers. Among such polymers one can cite polysaccharides of low water solubility, polylactides and polyglycolides and their copolymers, copolymers of lactides and lactones such as e-caprolactone, δ-valerolactone, polypeptides, and proteins such as gelatin, collagen, globulins and albumins. The great versatility in the selection of synthetic polymers is another advantage of the present invention since, as with allergic patients, one may wish to avoid using microballoons made of natural proteins (albumin, gelatin) like in US-A-4,276,885 or EP-A-324.938. Other suitable polymers include poly-(ortho)esters (see for instance US-A-4,093,709; US-A-4,131,648; US-A-4,138,344; US-A-4,180,646); polylactic and polyglycolic acid and their copolymers, for instance DEXON (see J. Heller, Biomaterials 1 (1980), 51; poly(DL-lactide-co-ε-caprolactone), poly(DL-lactide-co-δ-valerolactone), poly(DL-lactide-co-γ-butyrolactone), polyalkylcyanoacrylates; polyamides, polyhydroxybutyrate; polydioxanone; poly-β-aminoketones (Polymer 23 (1982), 1693); polyphosphazenes (Science 193 (1976), 1214); and polyanhydrides. References on biodegradable polymers can be found in R. Langer et al., Macromol. Chem. Phys. C23 (1983), 61-126. Polyamino-acids such as polyglutamic and polyaspartic acids can also be used as well as their derivatives, i.e. partial esters with lower alcohols or glycols. One useful example of such polymers is poly-(l.butyl-glutamate). Copolymers with other amino-acids such as methionine, leucine, valine, proline, glycine, alanine, etc. are also possible. Recently some novel derivatives of polyglutamic and polyaspartic acid with controlled biodegradability have been reported (see WO87/03891; US 4,888,398 and EP-130.935 incorporated here by reference). These polymers (and copolymers with other amino-acids) have formulas of the following type:

$$-(NH\text{-}CHA\text{-}CO)_x(NH\text{-}CHX\text{-}CO)_y$$

where X designates the side chain of an amino-acid residue and A is a group of formula $-(CH_2)_nCOOR^1R^2$-OCOR (II), with R^1 and R^2 being H or lower alkyls, and R being alkyl or aryl; or R and R^1 are connected together by a substituted or unsubstituted linking member to provide 5- or 6- membered rings.

A can also represent groups of formulas:

$$-(CH_2)_nCOO\text{-}CHR^1COOR \qquad (I)$$

and

$$-(CH_2)_nCO(NH\text{-}CHX\text{-}CO)_m\text{-}NH\text{-}CH(COOH)\text{-}(CH_2)_pCOOH \qquad (III)$$

and corresponding anhydrides. In all these formulas n, m and p are lower integers (not exceeding 5) and x and y are also integers selected for having molecular weights not below 5000.

The aforementioned polymers are suitable for making the microballoons according to the invention and, depending on the nature of substituents R, R^1, R^2 and X, the properties of the membrane can be controlled, for instance, strength, elasticity and biodegradability. For instance X can be methyl (alanine), isopropyl (valine), isobutyl (leucine and isoleucine), benzyl (phenylalanine).

Additives can be incorporated into the polymer wall of the microballoons to modify the physical properties such as dispersibility, elasticity and water permeability. For incorporation in the polymer, the additives can be dissolved in the polymer carrying phase, e.g. the hydrophobic phase to be emulsified in the water phase, whereby they will co-precipitate with the polymer during inter-facial membrane formation.

Among the useful additives, one may cite compounds which can "hydrophobize" the microballoons membrane in order to decrease water permeability, such as fats, waxes and high molecular-weight hydrocarbons. Additives which improve dispersibility of the microballoons in the injectable liquid-carrier are amphipatic compounds like the phospholipids; they also increase water permeability and rate of biodegradability.

Non-biodegradable polymers for making microballoons to be used in the digestive tract can be selected from most water-insoluble, physiologically acceptable, bioresistant polymers including polyolefins (polystyrene), acrylic resins (polyacrylates, polyacrylonitrile), polyesters (polycarbonate), polyurethanes, polyures and their copolymers. ABS (acryl-butadienestyrene) is a preferred copolymer.

Additives which increase membrane elasticity are the plasticizers like isopropyl myristate and the like. Also, very useful additives are constituted by polymers akin to that of the membrane itself but with relatively low molecular weight. For instance when using copolymers of polylactic/polyglycolic type as the membrane forming material, the properties of the membrane can be modified advantageously (enhanced softness and biodegradability) by incorporating, as additives, low molecular weight (1000 to 15,000 Dalton) polyglycolides or polylactides. Also polyethylene glycol of moderate to low M_w (e.g. PEG 2000) is a useful softening additive.

The quantity of additives to be incorporated in the polymer forming the inter-facially deposited membrane of the present microballoons is extremely variable and depends on the needs. In some cases no additive is used at all; in other cases amounts of additives which may reach about 20% by weight of the polymer are possible.

The injectable microballoons of the present invention can be stored dry in the presence or in the absence of additives to improve conservation and prevent coalescence. As additives, one may select from 0.1 to 25% by weight of water-soluble physiologi-

cally acceptable compounds such as mannitol, galactose, lactose or sucrose or hydrophilic polymers like dextran, xanthan, agar, starch, PVP, polyglutamic acid, polyvinylalcohol (PVA), albumin and gelatin. The useful life-time of the microballoons in the injectable liquid carrier phase, i.e. the period during which useful echographic signals are observed, can be controlled to last from a few minutes to several months depending on the needs; this can be done by controlling the porosity of the membrane from substantial imperviousness toward carrier liquids to porosities having pores of a few nanometers to several hundreds of nanometers. This degree of porosity can be controlled, in addition to properly selecting the membrane forming polymer and polymer additives, by adjusting the evaporation rate and temperature in step (4) of the method of claim 17 and properly selecting the nature of the compound (or mixture of compounds) constituting the hydrophobic phase, i.e. the greater the differences in its partial pressure of evaporation with that of the water phase, the coarser the pores in the microballoons membrane will be. Of course, this control by selection of the hydrophobic phase can be further refined by the choice of stabilizers and by adjusting the concentration thereof in order to control the rate of water evaporation during the forming of the microballoons. All these changes can easily be made by skilled ones without exercizing inventiveness and need not be further discussed.

It should be remarked that although the microballoons of this invention can be marketed in the dry state, more particularly when they are designed with a limited life time after injection, it may be desirable to also sell ready preparations, i.e. suspensions of microballoons in an aqueous liquid carrier ready for injection or oral administration. This requires that the membrane of the microballoons be substantially impervious (at least for several months or more) to the carrier liquid. It has been shown in this description that such conditions can be easily achieved with the present method by properly selecting the nature of the polymer and the interfacial deposition parameters. Actually parameters have been found (for instance using the polyglutamic polymer (where A is the group of formula II) and cyclooctane as the hydrophobic phase) such that the porosity of the membrane after evaporation of the hydrophobic phase is so tenuous that the microballoons are substantially impervious to the aqueous carrier liquid in which they are suspended.

A preferred administrable preparation for diagnostic purposes comprises a suspension in buffered or unbuffered saline (0.9% aqueous NaCl; buffer 10 mM tris-HCl) containing 10^8-10^{10} vesicles/ml. This can be prepared mainly according to the directions of the Examples below, preferably Examples 3 and 4, using poly-(DL-lactide) polymers from the Company Boehringer, Ingelheim, Germany.

The following Examples illustrate the invention practically.

Example 1

One gram of polystyrene was dissolved in 19 g of liquid naphthalene at 100°C. This naphthalene solution was emulsified at 90-95°C into 200 ml of a water solution of polyvinyl alcohol (PVA) (4% by weight) containing 0.1% of Tween-40 emulsifier. The emulsifying head was a Polytron PT-3000 at about 10,000 rpm. Then the emulsion was diluted under agitation with 500 ml of the same aqueous phase at 15°C whereby the naphthalene droplets solidified into beads of less than 50 µm as ascertained by passing through a 50 µm mesh screen. The suspension was centrifugated under 1000 g and the beads were washed with water and recentrifugated. This step was repeated twice.

The beads were resuspended in 100 ml of water with 0.8 g of dissolved lactose and the suspension was frozen into a block at -30°C. The block was thereafter evaporated under about 0.5-2 Torr between about -20 and -10°C. Air filled microballoons of average size 5-10 µm and controlled porosity were thus obtained which gave an echographic signal at 2.25 and 7.5 MHz after being dispersed in water (3% dispersion by weight). The stability of the microballoons in the dry state was effective for an indefinite period of time; once suspended in an aqueous carrier liquid the useful life-time for echography was about 30 min or more. Polystyrene being non-biodegradable, this material was not favored for injection echography but was useful for digestive tract investigations. This Example clearly establishes the feasibility of the method of the invention.

Example 2

A 50:50 copolymer mixture (0.3 g) of DL-lactide and glycolide (Du Pont Medisorb) and 16 mg of egg-lecithin were dissolved in 7.5 ml of $CHCl_3$ to give solution (1).

A solution (2) containing 20 mg of paraffin-wax (M.P. 54-56°C) in 10 ml of cyclooctane (M.P. 10-13°) was prepared and emulsified in 150 ml of a water solution (0.13% by weight) of Pluronic F-108 (a block copolymer of ethylene oxide and propylene oxide) containing also 1.2 g of $CHCl_3$. Emulsification was carried out at room temperature for 1 min with a Polytron head at 7000 rpm. Then solution (1) was added under agitation (7000 rpm) and, after about 30-60 sec, the emulsifier head was replaced by a helical agitator (500 rpm) and stirring was continued for about 3 hrs at room temperature (22°C). The suspension was passed through a 50 µm screen and frozen to a block which was subsequently evaporated between -20 and 0°C under high-vacuum (catching trap -60 to -80°C).

There were thus obtained 0.264 g (88%) of air-filled microballoons stable in the dry state.

Suspensions of said microballoons in water (no stabilizers) gave a strong echographic signal for at least one hour. After injection in the organism, they biodegraded in a few days.

Example 3

A solution was made using 200 ml of tetrahydrofuran (THF), 0.8 g of a 50:50 DL-lactide/glycolide copolymer (Boehringer AG), 80 mg of egg-lecithin, 64 mg of paraffin-wax and 4 ml of octane. This solution was emulsified by adding slowly into 400 ml of a 0.1% aqueous solution of Pluronic F-108 under helical agitation (500 r.p.m.). After stirring for 15 min, the milky dispersion was evaporated under 10-12 Torr 25°C in a rotavapor until its volume was reduced to about 400 ml. The dispersion was sieved on a 50 µm grating, then it was frozen to -40°C and freeze-dried under about 1 Torr. The residue, 1.32 g of very fine powder, was taken with 40 ml of distilled water which provided, after 3 min of manual agitation, a very homogeneous dispersion of microballoons of average size 4.5 µm as measured using a particle analyzer (Mastersizer from Malvern). The concentration of microballoons (Coulter Counter) was about 2 x 10^9/ml. This suspension gave strong echographic signals which persisted for about 1 hr.

If in the present example, the additives to the membrane polymer are omitted, i.e. there is used only 800 mg of the lactide/glycolide copolymer in the THF-/octane solution, a dramatic decrease in cell-wall permeability is observed, the echographic signal of the dispersion in the aqueous carrier not being significantly attenuated after 3 days.

Using intermediate quantities of additives provided beads with controlled intermediate porosity and life-time.

Example 4

There was used in this Example a polymer of formula defined in claim 8 in which the side group has formula (II) where R^1 and R^2 are hydrogen and R is tert.butyl. The preparation of this polymer (defined as poly-POMEG) is described in US-A-4,888,398.

The procedure was like in Example 3, using 0.1 g poly-POMEG, 70 ml of THF, 1 ml of cyclooctane and 100 ml of a 0.1% aqueous solution of Pluronic F-108. No lecithin or high-molecular weight hydrocarbon was added. The milky emulsion was evaporated at 27°C/10 Torr until the residue was about 100 ml, then it was screened on a 80 µm mesh and frozen. Evaporation of the frozen block was carried out (0.5-1 Torr) until dry. The yield was 0.18 g because of the presence of the surfactant. This was dispersed in 10 ml of distilled water and counted with a Coulter Counter. The measured concentration was found to be 1.43 x 10^9 microcapsules/ml, average size 5.21 µm as determined with a particle analyzer (Mastersizer from Malvern). The dispersion was diluted 100 x, i.e. to give about 1.5 x 10^7 microspheres/ml and measured for echogenicity. The amplitude of the echo signal was 5 times greater at 7.5 MHz than at 2.25 MHz. These signals were reproducible for a long period of time.

Echogenicity measurements were performed with a pulse-echo system consisting of a plexiglas specimen holder (diameter 30 mm) with a 20 µm thick Mylar acoustic window, a transducer holder immersed in a constant temperature water bath, a pulser-receiver (Accutron M3010JS) with an external preamplifier with a fixed gain of 40 dB and an internal amplifier with gain adjustable from -40 to +40 dB and interchangeable 13 mm unfocused transducers. A 10 MHz low-pass filter was inserted in the receiving part to improve the signal to noise ratio. The A/D board in the IBM PC was a Sonotek STR 832. Measurements were carried out at 2.25, 3.5, 5 and 7.5 MHz.

If in the present Example, the polymer used is replaced by lactic-lactone copolymers, the lactones being γ-butyrolactone, δ-valerolactone or ε-caprolactone (see Fukuzaki et al., J. Biomedical Mater. Res. 25 (1991), 315-328), similar favorable results were obtained. Also in a similar context, polyalkylcyanoacrylates and particularly a 90:10 copolymer poly(DL-lactide-coglycolide) gave satisfactory results. Finally, a preferred polymer is a poly(DL-lactide) from the Company Boehringer-Ingelheim sold under the name "Resomer R-206"or Resomer R-207.

Example 5

Two-dimensional echocardiography was performed using an Acuson-128 apparatus with the preparation of Example 4 (1.43 x 10^9/ml) in an experimental dog following peripheral vein injection of 0.1-2 ml of the dispersion. After normally expected contrast enhancement imaging of the right heart, intense and persistent signal enhancement of the left heart with clear outlining of the endocardium was observed, thereby confirming that the microballoons made with poly-POMEG (or at least a significant part of them) were able to cross the pulmonary capillary circulation and to remain in the blood-stream for a time sufficient to perform efficient echographic analysis.

In another series of experiments, persistent enhancement of the Doppler signal from systemic arteries and the portal vein was observed in the rabbit and in the rat following peripheral vein injection of 0.5-2 ml of a preparation of microballoons prepared as disclosed in Example 4 but using poly(DL-lactic acid) as the polymer phase. The composition used contained 1.9 x 10^9 vesicles/ml.

Another composition prepared also according to the directions of Example 4 was achieved using po-

ly(tert.butyl-glutamate). This composition (0.5 ml) at dilution of 3.4×10^8 microballoons/ml was injected in the portal vein of rats and gave persistent contrast enhancement of the liver parenchyma.

Example 8

A microballoon suspension (1.1×10^9 vesicles/ml) was prepared as disclosed in Example 1 (resin = polystyrene). One ml of this suspension was diluted with 100 ml of 300 mM mannitol solution and 7 ml of the resulting dilution was administered intragastrically to a laboratory rat. The animal was examined with an Acuson-128 apparatus for 2-dimensional echography imaging of the digestive tract which clearly showed the single loops of the small intestine and of the colon.

Claims

1. Microcapsules or microballoons of micronic or submicronic size bounded by a polymer membrane filled with air or a gas suitable, when in the form of suspensions in a liquid carrier, to be administered to human or animal patients for therapeutic or diagnostic applications, e.g. for the purpose of ultrasonic echography imaging, characterized in that the polymer of the membrane is a deformable and resilient interfacially deposited polymer.

2. Air or gas filled microballoons bounded by an elastic interfacial polymeric membrane adapted to form with suitable physiologically acceptable aqueous carrier liquids suspensions to be taken orally, rectally and urethrally, or injectable into living organisms for therapeutic or diagnostic purposes, characterized in being non-coalescent dry and instantly dispersible by admixing with said liquid carrier.

3. The microballoons of claims 1 or 2 having size mostly in the 0.5 - 10 µm range suitable for injection into the blood-stream of living beings, characterized in that the membrane polymer is biodegradable and the membrane is either impervious or contains pores permeable to bioactive liquids for increasing the rate of biodegradation.

4. The microballoons of claim 3, in which the polymer membrane has a porosity ranging from a few nanometers to several hundreds or thousands of nanometers, preferably 50-2000 nm.

5. The microballoons of claim 3, in which the membrane is elastic, has a thickness of 50-600 nm, and resists pressure variations produced by heart beat pulsations in the blood-stream.

6. The microballoons of claim 3, in which the polymer of the membrane is a biodegradable polymer selected from polysaccharides, polyamino-acids, polylactides and polyglycolides and their copolymers, copolymers of lactides and lactones, polypeptides, poly-(ortho)esters, polydioxanone, poly-β-amino-ketones, polyphosphazenes, polyanhydrides and poly(alkyl-cyanoacrylates).

7. The microballoons of claim 3, in which the membrane polymer is selected from polyglutamic or polyaspartic acid derivatives and their copolymers with other amino-acids.

8. The microballoons of claim 7, in which the polyglutamic and polyaspartic acid derivatives are selected from esters and amides involving the carboxylated side function thereof, said side functions having formulae

$$-(CH_2)_nCOO-CHR^1COOR \quad (I),$$

or

$$-(CH_2)_nCOOR^1R^2-O-COR \quad (II),$$

or

$$-(CH_2)_nCO(NH-CHX-CO)_mNHCH(COOH)-(CH_2)_pCOOH \quad (III),$$

in which R is an alkyl or aryl substituent; R^1 and R^2 are H or lower alkyls, or R and R^1 are connected together by a substituted or unsubstituted linking member to form a 5- or 6-membered ring; n is 1 or 2; p is 1, 2 or 3; m is an integer from 1 to 5 and X is a side chain of an aminoacid residue.

9. The microballoons of claim 3, in which the membrane polymer contains additives to control the degree of elasticity, and the size and density of the pores for permeability control.

10. The microballoons of claim 9, in which said additives include plasticizers, amphipatic substances and hydrophobic compounds.

11. The microballoons of claim 10, in which the plasticizers include isopropyl myristate, glyceryl monostearate and the like to control flexibility, the amphipatic substances include surfactants and phospholipids like the lecithins to control permeability by increasing porosity and the hydrophobic compounds include high molecular weight hydrocarbon like the paraffinwaxes to reduce porosity.

12. The microballoons of claim 10, in which the additives include polymers of low molecular weight, e.g. in the range of 1000 to 15.000, to control softness and resiliency of the microballoon mem-

brane.

12. The microballoons of claim 12, in which the low molecular weight polymer additives are selected from polylactides, polyglycolides, polyalkylene glycols like polyethylene glycol and polypropylene glycol, and polyols like polyglycerol.

14. The microballoons of claims 1 or 2, having size up to about 1000 µm suitable for oral, rectal and urethral applications, characterized in that the membrane polymer is not biodegradable in the digestive tract and impervious to biological liquids.

15. The microballoons of claim 14, in which the polymer is selected from polyolefins, polyacrylates, polyacrylonitrile, non-hydrolyzable polyesters, polyurethanes and polyureas.

16. Aqueous suspension of the microballoons according to claims 1 or 2 for administration to patients, characterized in containing a concentration of about 10^6 to 10^{10} microballoons/ml, this being stable for a period exceeding a month.

17. A method for making air or gas filled microballoons usable as suspensions in a carrier liquid for oral, rectal and urethral applications, or for injections into living organisms, this method comprising the steps of:

(1) emulsifying a hydrophobic organic phase into a water phase so as to obtain droplets of said hydrophobic phase as an oil-in-water emulsion in said water phase;

(2) adding to said emulsion a solution of at least one polymer in a volatile solvent insoluble in the water phase, so that a layer of said polymer will form around said droplets;

(3) evaporating said volatile solvent so that the polymer will deposit by interfacial precipitation around the droplets which then form beads with a core of said hydrophobic phase encapsulated by a membrane of said polymer, said beads being in suspension in said water phase;

(4) subjecting said suspension to reduced pressure under conditions such that said encapsulated hydrophobic phase be removed by evaporation;

characterized in that said hydrophobic phase is selected so that in step (4) it evaporates substantially simultaneously with the water phase and is replaced by air or gas, whereby dry, free flowing, readily dispersible microballoons are obtained.

18. The method of claim 17, in which said polymer is dissolved in said hydrophobic phase, so that steps (2) and (3) can be omitted and the polymer membrane will form by interfacial precipitation during step (4).

19. The method of claim 17, characterized in that evaporation of said hydrophobic phase in step (4) is performed at a temperature where the partial vapour pressure of said hydrophobic phase is of the same order as that of water vapour.

20. The method of claim 17, in which said evaporation of step (4) is carried out under freeze-drying conditions.

21. The method of claim 20, in which freeze-drying is effected at temperatures of from -40°C to 0°C.

22. The method of claims 17 or 19, in which the hydrophobic phase is selected from organic compounds having a vapour pressure of about 1 Torr at a temperature comprised in the interval of about -40°C to 0°C.

23. The method of claims 17 or 18, in which the aqueous phase comprises, dissolved, from about 1 to 20% by weight of stabilizers comprising hydrophilic compound selected from sugars, PVA, PVP, gelatin, starch, dextran, polydextrose, albumin and the like.

24. The method of claim 18, in which additives to control the degree of permeability of the microballoons membrane are added to the hydrophobic phase, the rate of biodegradability of the polymer after injecting the microballoons into living organisms being a function of said degree of permeability.

25. The method of claim 24, in which the said additives include hydrophobic solids like fats, waxes and high molecular weight hydrocarbons, the presence of which in the membrane polymer of the microballoons will reduce permeability toward aqueous liquids.

26. The method of claim 24, in which the said additives include amphipatic compounds like the phospholipids, or low molecular weight polymers, the presence of which in the membrane polymer will increase permeability of the microballoons to aqueous liquids.

27. The method of claim 18, in which the hydrophobic phase subjected to emulsification in said water phase also contains a water-soluble solvent which, upon being diluted into said water phase during emulsification, will aid in reducing the size

of droplets and induce interfacial precipitation of the polymer before step (4) is carried out.

28. A method for making air or gas filled microballoons usable as suspensions in a carrier liquid for oral, rectal and urethral applications, or for injections into living organisms, this method comprising the steps of:

(1) emulsifying a hydrophobic organic phase into a water phase so as to obtain droplets of said hydrophobic organic phase as an oil-in-water emulsion in said water phase, said organic phase containing, dissolved therein, one or more water-insoluble polymers;

(2) subjecting said emulsion to reduced pressure under conditions such that said hydrophobic phase be removed by evaporation, whereby the polymer dissolved in the droplets will deposit interfacially and form a polymer bounding membrane, the droplets being simultaneously converted to microballoons,

characterized in that said hydrophobic phase is selected so that in step (2) it evaporates substantially simultaneously with the water phase and, upon evaporation, is replaced by air or gas, whereby the microballoons obtained are in dry, free flowing and readily dispersible form.

29. The method of claim 28, in which the hydrophobic polymer solution phase subjected to emulsification in said water phase also contains a water-soluble solvent which, upon being diluted into said water phase during emulsification, will aid in reducing the size of droplets and induce interfacial precipitations of the polymer before step (2) is carried out.

30. The method of claim 28, in which said organic hydrophobic phase emulsified in step (1) contains no polymer dissolved therein, and before carrying through step (2), the following additional steps are performed:

(1a) adding to said emulsion a solution of at least one polymer in a volatile solvent insoluble in the water phase, so that a layer of said polymer will form around said droplets;

(1b) evaporating said volatile solvent so that the polymer will deposit by interfacial precipitation around the droplets, thus forming microballoons or beads with a core of said hydrophobic phase encapsulated by a membrane of said polymer, said beads being in suspension in said water phase, whereby in step (2) evaporation of said hydrophobic phase takes place through said membrane and provides it with substantial microporosity.

31. An injectable aqueous suspension of microballoons containing 10^8-10^{10} vesicles/ml bounded by a membrane of interfacially precipitated DL-lactide polymer defined by the commercial name of Resomer.

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EUROPEAN SEARCH REPORT

Application Number

EP 91 81 0366
Page 1

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	CHEMICAL ABSTRACTS, vol. 111, no. 24, 27 November 1989 Columbus, Ohio, USA M. FESSI et al.: ""Nanocapsule formation by interfacial polymer deposition following solvent displacement" & Int. J. Pharm. 1989, vol.55, no.1, pages 81-84" page 314; column 2; ref. no. 219235Z " abstract "	1-3, 6, 17, 28	A61K49/00 A61K9/00
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The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	16 AUGUST 1991	ALVAREZ ALVAREZ C.

CATEGORY OF CITED DOCUMENTS

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DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A B	WO-A-8002365 (RASOR ASSOCIATES INC.) * pages 13 - 14 * & US-A-4276885	1-4	
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A	DATABASE BIOCHEMICAL ABSTRACTS AN-85:355072 vol.80, abstract no.25064, 1985, Philadelphia,US Y.OHARA et al.:"Preparation of ethylcellulose-polystyrene composite microcapsules..." & J. Membr. Sci. 1985 vol.23 no.1 pages 1-10 * whole abstract *	1, 2, 14, 15	
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